

DISSERTATION

Untersuchungen zur antineoplastischen Wirkung neuartiger
HDAC-Inhibitoren bei Prostata- und hepatozellulären
Karzinomen

Studies on the antineoplastic effect of novel HDAC inhibitors in
prostate and hepatocellular carcinomas

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Abkürzungsverzeichnis

Adenosindiphosphat (ADP)

Adenosintriphosphat (ATP)

Alpha-Fetoprotein (AFP)

Androgen Rezeptor (AR, flAR = Full Length Androgen Rezeptor)

Androgendeprivationstherapie (ADT)

Antiandrogenen Zweitlinientherapeutikums (2ndLAARx)

Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften e. V. (AWMF)

Aspartat-Aminotransferase (AST)

B cell lymphoma-2-like 11 (BCL2L11))

Barcelona Clinic Liver Cancer (BCLC)

Bicinchoninsäure (BCA)

Bovines Serumalbumin (BSA)

Chorioallantoismembran (CAM)

Desoxyribonukleinsäure (DNA)

Dimethylsulfoxid (DMSO)

Dosislimitierender Toxizität (DLT)

Enzyme-linked Immunosorbent Assay (ELISA)

Epidermal Growth Factor Receptor (EGFR)

Ethylendiamintetraessigsäure (EDTA)

European Association for the Study of the Liver (EASL)

Fetalem Kälberserum (FKS)

Fläche unter der Kurve (area under the curve = AUC)

Food and Drug Administration (FDA)

Gesamtüberleben (overall survival = OS)

Gonadotropin-Releasing-Hormon (GnRH)

Grad Celsius (°C)

Hepatitis B (HBV)

Hepatitis C (HCV)

Hepatozelluläre Karzinom (HCC)

High-Dose-Rate-Brachytherapie (HDR-Brachytherapie)

Histonacetylase (HAT)

Histondeacetylase (HDAC)
Histondeacetylase-Inhibitoren (HDAC-Inhibitoren)
Immunoreactivity score (IRS)
International Diabetes Federation (IDF)
International Society of Urological Pathology (ISUP)
Kohlenstoffdioxid (CO₂)
Komplette Remission (complete response = CR)
Krankheits-Kontroll Rate (disease control rate = DCR)
Krankheitsfreien Überlebensraten (disease-free survival (DFS))
Kutanen T-Zell-Lymphoms (CTCL)
Laktatdehydrogenase (LDH)
Low-Dose-Rate-Brachytherapie (LDR-Brachytherapie)
Magnetresonanztomographie (MRT)
Maximal tolerierbaren Dosis (MTD)
metastasierten, kastrationsresistenten Prostatakarzinoms (mCRPC)
Mikrometer (µm)
Mikromolar (mM)
Mittlere inhibitorische Konzentration (IC₅₀)
Multiplen Myelomen (MM)
Nanometer (nm)
Nanomolar (nM)
National Comprehensive Cancer Network (NCCN)
Nicht-kleinzellige Lungenkarzinom (NSCLC)
Nichtalkoholische Fettleber (non-Alcoholic Fatty Liver Disease = NAFLD)
Nicotin-Amid-Adenin-Dinukleotid (NAD⁺)
Partielles Ansprechen (Partial Response = PR)
PD-L1 (programmed death-ligand 1)
peripheren T-Zell Lymphomen (PTCL)
Phosphatgepufferter Salzlösung (PBS)
Plattenepithelkarzinomen von Kopf und Hals (head and neck squamous cell carcinoma = HNSCC)
Poly(ADP-Ribose)-Polymerasen (PARP)
Polyvinylidendifluorid-Membranen (PVDF)
Progression (time to progression = TTP)

Progressionsfreies Überleben (progression free survival = PFS)
Prostatakarzinom (PCa)
Prostata-spezifisches Antigen (PSA)
Prostate Cancer Clinical Trials Working Group 2 (PCGW2)
Radioimmunpräzipitationspuffer (RIPA)
Radiologisch progressionsfreies Überleben (rPFS)
Radiologische progressionsfrei (rPF)
Reaktive Sauerstoffspezies (ROS)
Response Evaluation Criteria in Solid Tumors criteria (RECIST Kriterien)
Robert Koch Institut (RKI)
Roswell Park Memorial Institute (RPMI)
Sirtuine (SIRT 1-7, silent information regulator 2 (Sir2) proteins)
Stabile Erkrankung (stable disease (SD))
Ständigen Impfkommission (STIKO)
Transarteriellen Chemoembolisation (TACE)
Umdrehungen pro Minute (revolutions per minute = Rpm)
Vascular Endothelial Growth Factor (VEGF)
Wasserstoffperoxid (H₂O₂)
World Health Organisation (WHO)

Zusammenfassung

Die neu entwickelten Substanzen 3BrQuin-SAHA, 3CIQuin-SAHA, zwei chimäre EGFR/HDAC-Inhibitoren, und SF5-SAHA, ein HDAC-Inhibitor, wurden in ihrer Wirkung und Wirkmechanismen an Zellen des Prostata- (PCa) und hepatozellulären (HCC) Karzinoms genauer charakterisiert. Eine Verbesserung der Therapie beider Tumorentitäten ist, gemessen am Auftreten weltweit letal verlaufender Fälle, klinisch von äußerster Relevanz und anzustreben.

In Zellproliferationsversuchen hemmten die neuartigen Inhibitoren das Wachstum von DU145- (PCa) und Hep-G2 (HCC) Zellen im einstelligen mikromolaren Bereich. Als zugrundeliegender Wirkmechanismus für die beobachteten antiproliferativen Effekte der Inhibitoren konnte durch Bestimmung der Laktatdehydrogenase im Überstand unspezifische Zytotoxizität ausgeschlossen werden und durch Kinase-Assays die Target-spezifische Wirkung der Substanzen belegt werden. 3BrQuin-SAHA und 3CIQuin-SAHA hemmten die Epidermal Growth Factor Receptor (EGFR)-Kinase in einem vergleichbaren Ausmaß wie der reine EGFR-Inhibitor Gefitinib und alle getesteten Substanzen reduzierten die Histon-Deacetylase (HDAC)-Aktivität ähnlichen stark wie der HDAC-Inhibitor SAHA. Als weiter Marker für einen gezielten Zelluntergang konnte eine Induktion der apoptosespezifischen Caspase-3-Aktivität gemessen werden und eine vermehrte Bildung von reaktiven Sauerstoffspezies qualitativ bestimmt werden. Western Blot Untersuchungen an DU145 Zellen zeigten eine dosisabhängig gesteigerte Acetylierung von Histon H3, eine verminderte Expression des EGFR, sowie eine durch Caspase-3 induzierte gesteigerte Spaltung der Poly(ADP-Ribose)-Polymerasen (PARP). Im Scratch-Assay wurde zudem gezeigt, wie 3CIQuin-SAHA und SF5-SAHA die Migration von DU145 Zellen verlangsamt. Weiterführend wurden in vivo die antiangiogenetischen Effekte der Inhibitoren an der stark vaskularisierten Chorioallantoismembran (CAM) evaluiert und aufgezeigt. Die vielversprechenden Ergebnisse legen nahe, dass HDAC-Inhibitoren als Einzelsubstanzen oder als duale Inhibitoren in Form von EGFR/HDAC-Chimära eine näher zu untersuchende Therapiealternative darstellen und weitere Versuche zum besseren Verständnis der Relevanz und Wirkweise von HDAC-Inhibitoren zur Therapie von PCa und HCC anzustreben sind.

Abstract

The newly developed substances 3BrQuin-SAHA, 3ClQuin-SAHA, two chimeric EGFR/HDAC inhibitors, and SF5-SAHA, an HDAC inhibitor, were characterized in more detail in terms of their effect and mechanisms of action on cells of prostate (PCa) and hepatocellular (HCC) carcinoma. An improvement in the therapy of both tumor types, measured by the incidence of lethal cases worldwide, is clinically of utmost relevance and should be strived for.

In cell proliferation experiments, the novel inhibitors restrain the growth of DU145 (PCa) and Hep-G2 (HCC) cells in a single-digit micromolar range. The underlying mechanism of action for the observed antiproliferative effects of the inhibitors was ruled out by determination of lactate dehydrogenase in the supernatant and the target-specific effect of the substances was proven by kinase assays. 3BrQuin-SAHA and 3ClQuin-SAHA inhibited epidermal growth factor receptor (EGFR) kinase to a comparable extent as the pure EGFR inhibitor gefitinib and all tested substances reduced histone deacetylase (HDAC) activity to a similar extent as the HDAC inhibitor SAHA. As a further marker for targeted cell death, an induction of apoptosis-specific caspase-3 activity was measured, and an increased formation of reactive oxygen species was qualitatively determined. Western blot investigations on DU145 cells showed a dose-dependent increased acetylation of histone H3, a decreased expression of EGFR, as well as an increased cleavage of poly(ADP-ribose) polymerases (PARP) induced by caspase-3. Scratch assays also showed how 3ClQuin-SAHA and SF5-SAHA slow down the migration of DU145 cells. Further, the anti-angiogenic effects of the inhibitors were evaluated and demonstrated in vivo at the highly vascularised chorioallantoic membrane (CAM).

The promising results suggest that HDAC inhibitors as single compounds or as dual inhibitors in the form of EGFR/HDAC chimera represent a therapeutic alternative to be investigated further and that further studies to better understand the relevance and mode of action of HDAC inhibitors for the therapy of PCa and HCC should be pursued.

1. Einleitung

Die durchschnittliche Lebenserwartung eines Neugeborenen in Deutschland beträgt laut der letzten Berechnung der World Health Organisation (WHO) 81,72 Jahre [1]. Dieser demografische Wandel trägt neben anderen sozioökonomischen Faktoren maßgeblich dazu bei, dass Tumorerkrankungen die zweithäufigste Todesursache weltweit darstellt [2, 3]. In Ländern mit sehr hohem Index für menschliche Entwicklung, ein zusammengesetztes Maß aus Lebenserwartung, Einkommen und Zugang zu Bildung, sind Tumorerkrankungen bereits jetzt die Hauptursache für den vorzeitigen Todeseintritt und werden in den kommenden Jahren auch in anderen Ländern noch mehr an Bedeutung gewinnen [4]. Im Jahr 2020 führten schätzungsweise 19,3 Millionen neue Tumorerkrankungen zu 10 Millionen tumorassoziierten Toden weltweit [2]. Angesichts der enormen Relevanz für das Gesundheitssystem gilt es neuartige und bessere Therapieansätze für die Bekämpfung von Tumorkrankheiten zu erforschen.

1.1 Prostatakarzinom

Das Prostatakarzinom (PCa) ist das am zweithäufigsten auftretende Karzinom der männlichen Weltbevölkerung und macht mit 7,3 % den viertgrößten Anteil der Karzinome aller Geschlechter aus [2]. Trotz der verhältnismäßig hohen Fünf-Jahres-Überlebensrate von 61,9 %, steht das Prostatakarzinom bei Männern an fünfter Stelle der durch Karzinome verursachten Tode [2, 5]. Das Robert Koch Institut (RKI) ermittelte für das Jahr 2018 in Deutschland ein mittleres Erkrankungsalter von 71 Jahren, wodurch gleichzeitig der wichtigste Risikofaktor, nämlich Alter, beschrieben wird [6]. Weitere wichtige Faktoren sind eine positive Familienanamnese, da bei Verwandten ersten Grades ein 2,5-fach erhöhtes Risiko besteht an einem PCa zu erkranken, sowie der Lebensort [7, 8]. Die höchsten Inzidenzen bestehen in Nord- und Westeuropa, der Karibik, Australien/Neuseeland, Nordamerika sowie Südafrika und die niedrigsten in Asien und Nordafrika [2]. Zahlreiche Keimbahnmutationen wurden als Risikofaktoren für die Entstehung von PCa ausgemacht [9]. Als relevanteste dieser haben Patienten mit *BRCA2* Mutation ein höheres Erkrankungsrisiko mit aggressiveren Verläufen im Vergleich zu nicht Trägern [10, 11]. Wie aus Abbildung 1 zu entnehmen, wird die Prostata anatomisch in fünf Zonen unterteilt, wobei die periphere Zone 70 % und die zentrale Zone 25 % des Organvolumens ausmachen.

Die Mehrheit der PCa entstehen aus der peripheren Zone, während die benigne Prostat hyperplasie vor allem aus einer Volumenzunahme der Transitionszone resultiert [12].

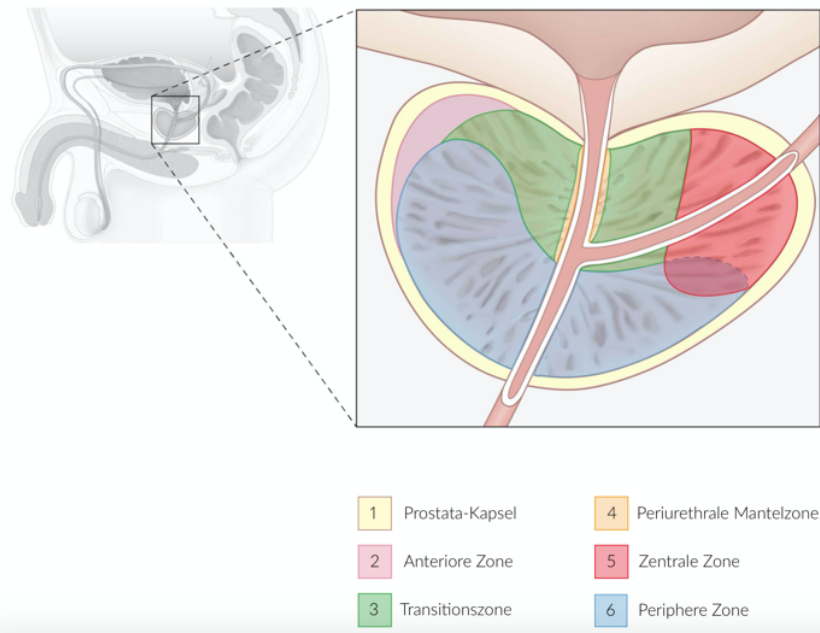


Abbildung 1: Einteilung der Prostata in fünf Zonen nach McNeal [13].

1.1.1 Diagnostik und Staging

Obwohl der Nutzen von Screenings mittels dem Tumormarker prostataspezifisches Antigen (PSA) zur PCa Früherkennung heterogen diskutiert wird, empfiehlt die deutsche Leitlinie der Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften e. V. (AWMF) folgendes Vorgehen. Ab dem 45. Lebensjahr und einer Lebenserwartung von > 10 Jahren kann, neben Anamnese zu Beschwerden oder gesundheitlichen Veränderungen und der Untersuchung der äußeren Geschlechtsorgane sowie Lymphknoten, das Angebot einer PSA-Bestimmung unterbreitet werden. Unbedingt sollte eine ausführliche Beratung zum Nutzen und Aussagekraft der Bestimmung im Vorhinein erfolgen. Ergänzend zur PSA-Bestimmung kann eine digital-rektale Untersuchung empfohlen werden. Bei einem wiederholt gemessenem PSA Wert von ≥ 4 ng/ml, einem PSA Anstieg zwischen 0,35 ng/ml und 0,75 ng/ml pro Jahr oder auffälligen Tastbefunden sollte eine Prostatabiopsie, nach eventueller vorangegangener Bildgebung mittels Magnetresonanztomographie (MRT) und transrektalem Ultraschall (TRUS), empfohlen werden [14]. Die gewonnenen Gewebeproben (Stanzen) werden anhand ihres Entartungsmusters, nach dem von Donald Gleason etablierten Grading-Systems, in 5 Stufen eingeteilt (Abbildung 2). Der zugehörige Gleason-Score bildet die Summe des am häufigsten und des am schlechtesten differenzierten Gleason-Grades ab, bzw. bei Prostatektomiepräparat

die des am häufigsten, mit dem am zweithäufigsten vorkommenden Entdifferenzierungsgrades [15]. Basierend auf diesem System führte die International Society of Urological Pathology (ISUP) im Jahr 2014 ein modifiziertes System aus fünf „Grade Groups“ zur besseren Einteilung ein (Tabelle 1) [16].

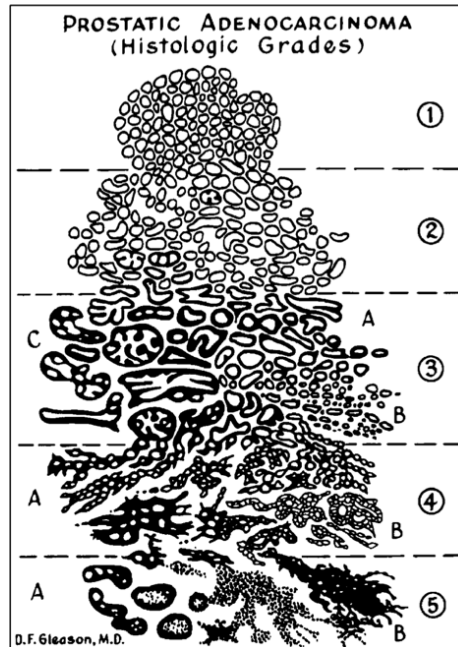


Abbildung 2: Einteilung des Prostatakarzinoms anhand des Gleason-Scores. Skizzenhafte Darstellung der fünf Entartungsgrade nach D.F. Gleason [15].

Tabelle 1: Neues Graduierungssystem der ISUP zur Einteilung des Prostatakarzinoms.

Grad-gruppe	Gleason-Score	
1	≤ 6	Nur einzelne diskrete, gut ausgebildete Drüsen
2	3 + 4 = 7	Überwiegend gut geformte Drüsen mit einem geringeren Anteil schlecht geformter/verschmolzener/kribriiformer Drüsen
3	4 + 3 = 7	Überwiegend schlecht geformte/verschmolzene/kribriiforme Drüsen mit einem geringen Anteil an gut geformten Drüsen
4	4 + 4 = 8	Nur schlecht ausgebildete/verschmolzene/kribriiforme Drüsen oder
	3 + 5 = 8	Überwiegend gut ausgebildete Drüsen und ein kleinerer Teil ohne Drüsen oder
5	5 + 3 = 8	Überwiegend fehlende Drüsen und geringer Anteil an gut ausgebildeten Drüsen
	9 - 10	Fehlende Drüsenbildung (oder bei Nekrosen) mit oder ohne schlecht geformten/verschmolzenen/kribriiformen Drüsen

Modifiziert aus [16].

Zum Staging, also der Beschreibung von Tumorgröße und Ausbreitungsmuster, wird die TNM-Klassifikation verwendet. Als lang etabliertes und immer noch häufig verwendetes System zur Risikostratifizierung wird die Klassifikation nach D'Amico, welche Tumore in niedriges, intermediäres oder hohes Risiko einteilt, je nach PSA-Level, Gleason Score und cT-Kategorie, genutzt (Tabelle 2) [17]. Neure Klassifikationen, wie die des National Comprehensive Cancer Network (NCCN), unterteilen zusätzlich in sehr niedriges, intermediär günstiges bzw. ungünstiges und sehr hohes Risiko [18].

Tabelle 2: System zur Risikostratifizierung von Prostatakarzinomen nach D'Amico

Risikoeinteilung	Parameter
niedriges Risiko	PSA \leq 10 ng/mL und Gleason-Score 6 (Gleason Grade Group I) und cT-Kategorie 1c, 2a
intermediäres Risiko	PSA $>$ 10 bis \leq 20 ng/mL oder Gleason-Score 7 (Gleason Grade Group II und III) oder cT-Kategorie 2b
hohes Risiko	PSA $>$ 20 ng/mL oder Gleason-Score \geq 8 (Gleason Grade Group IV und V) oder cT-Kategorie 2c

Aus [19].

1.1.2 Therapie

Zur Therapie des Prostatakarzinoms stehen verschiedene Optionen zur Verfügung [14]. Grundlegend wird hierbei zwischen kurativen und beobachtenden Therapieverfahren unterschieden. Um einer Übertherapie entgegenzuwirken, d. h. Tumorthherapie ohne Einfluss auf die Lebenserwartung, müssen Indikation für kurative Intervention besonders kritisch gestellt werden und gemeinsam mit den Patienten erörtert werden. Laut AWMF-Leitlinien kommen für das Therapiekonzept der aktiven Überwachung Patienten mit lokal begrenzten Tumoren, die folgende Charakteristika erfüllen, PSA \leq 10 ng/ml, Gleason \leq 6, cT1 und cT2a, Tumor in \leq 2 Stenzen bei leitliniengerechter Entnahme von 10 bis 12 Stenzen, \leq 50 % Tumor in einer Stanze, in Betracht. Ziel ist es, die Zahl der „Übertherapien“ zu verringern, aber durch ein definiertes Kontroll- und Überwachungsschema den richtigen Zeitpunkt für eine aktive Therapie bei Tumorprogression abzuspannen. Bei Patienten mit einer Lebenserwartung von $<$ 10 Jahren und nicht metastasierten Tumoren mit initialem Gleason-Score \leq 7 kann auf aktive Überwachung des Tumors verzichtet werden und im Rahmen des „Watchfull Waiting“ Konzepts ausschließlich symptomorientiert behandelt werden [14].

Als kurative primäre Therapieoptionen werden laut aktueller S3 AWMF-Leitlinie gleichwertig die radikale Prostatektomie und die perkutane Strahlentherapie, mit eventueller

adjuvanter hormonablativer Therapie bei mittlerem bis hohem Risikoprofil, bei lokal begrenzten und fortgeschrittenen Karzinomen, sowie eine Low-Dose-Rate-Brachytherapie (LDR-Brachytherapie) für lokal begrenzte Karzinome mit niedrigem Risikoprofil, empfohlen. Eine weitere Therapieoption bietet die Kombination aus High-Dose-Rate-Brachytherapie (HDR-Brachytherapie) plus perkutaner Strahlentherapie. Nach radikaler Prostatektomie wird nur für bestimmte Patientengruppe eine adjuvante Strahlentherapie empfohlen (u. a. positiver Schnittrand) [14].

Zur systemischen Therapie des metastasierten hormonsensitiven Prostatakarzinoms (mHSPC) stehen mehrere Medikamente sowie die beidseitige Orchiektomie zur Androgendeprivation zur Verfügung. Zu den relevanten Medikamenten gehören GnRH-Agonisten (Leuproreline, Busereline, Gosereline, Triptoreline), GnRH-Antagonisten (Abarelix, Degarelix) und nichtsteroidale oder steroidale Antiandrogene (Bicalutamid, Flutamid, Cyproteronacetat) [20]. Die antiandrogene Therapie kann darüber hinaus mit den mitosehemmenden Taxan-Chemotherapeutika wie Docetaxel und Cabazitaxel (zweite Wahl) oder Hormonpräparaten der neuen Generation (Abirateron, Apalutamid und Enzalutamid) kombiniert werden. Selbige Chemotherapeutika und Hormonpräparate der neuen Generation werden zur Therapie des metastasierten, kastrationsresistenten Prostatakarzinoms (mCRPC) eingesetzt [14].

Zusammenfassend lässt sich sagen, dass das lokal begrenzte Prostatakarzinom eine gute Prognose hat. Aus der Cochrane Analyse aus dem Jahr 2020, die radikale Prostatektomie mit „Watchful waiting“ vergleicht, ist zu entnehmen, dass nach 20 Jahren gerade mal ungefähr 10 % der Patienten mit lokal begrenzten Prostatakarzinom (T1-2, N0, M0) ohne Intervention an einem mit Prostata Krebs assoziiertem Tod verstarben [21]. Trotzdem verstarben im Jahr 2020 schätzungsweise 375 304 Menschen an Prostatakarzinom [2]. Patienten, für die es keinen kurativen Therapieansatz gibt, ist die androgendeprivative Therapie zu wählen [14]. Nach initialem Therapieansprechen wird jedoch häufig ein Fortschreiten der Erkrankung beobachtet. Man spricht von kastrationsresistenten Tumoren, die mit einer schlechteren Prognose einhergehen [22]. Um eine bessere Versorgung von Patienten mit PCa in fortgeschrittenem Stadium zu sichern, gilt es alternativen und bessere Therapieoptionen zu erforschen.

1.2 Hepatozelluläres Karzinom (HCC)

Leberkarzinome stellen die sechsthäufigste Tumorentität (4,7 %) weltweit dar, machen jedoch die dritthäufigste karzinombezogene Todesursache aus (8,3 %). Das hepatozelluläre Karzinom (HCC) repräsentiert mit 75 - 85 % den größten Anteil der Leberkarzinome [2]. In Deutschland beträgt das mittlere Erkrankungsalter laut den aktuellen Zahlen des RKIs aus dem Jahr 2018, 75 Jahre für Frauen und 71 Jahre für Männer [23]. Die wichtigsten Risikofaktoren für die Entwicklung eines HCC sind chronische Hepatitis B (HBV) und Hepatitis C (HCV) Virusinfektionen sowie Alkohol [24]. Weitere Faktoren, die das Risiko erhöhen, an einem HCC zu erkranken, sind der Verzehr von Aflatoxin kontaminierten Lebensmitteln und Rauchen, vor allem als Kofaktor bei bestehender HBV-Infektion [25, 26]. Seltene Erkrankungen wie Hämochromatose, Porphyrie und Alpha-1-Antitrypsinmangel erhöhen das Risiko an HCC zu erkranken [27-30]. Aufgrund des Wandels des Lifestyles in der heutigen Gesellschaft rücken andere Risikofaktoren weiter in den Vordergrund. Laut der International Diabetes Federation (IDF) stieg die weltweite altersadaptierte Prävalenz von Diabetes von 2000 bis 2021 von 4,6 auf 9,8 % [31]. Diabetes steigert das Risiko an HCC zu erkranken als isolierter Faktor, sowie als Kofaktor bei Patient:innen mit chronischer HBV Infektion [32, 33]. Nichtalkoholische Fettleber (non-Alcoholic Fatty Liver Disease = NAFLD) Erkrankungen steigern das Risiko für HCC. Besteht eine Zirrhose aufgrund der NAFLD ist das Risiko vergleichbar mit Patient:innen mit Leberzirrhose andere Genese. Ob die nichtalkoholische Fettleber ohne das Auftreten von Zirrhose das Risiko steigert, an einem HCC zu erkranken, wird unterschiedlich diskutiert [34, 35].

1.2.1 Diagnostik und Staging

Zur primären Prävention empfiehlt die aktuelle AWMF-Leitlinie die Impfung gegen HBV gemäß den Vorgaben der Ständigen Impfkommission (STIKO). Zur sekundären Prävention sollten risikobehaftete Grunderkrankungen behandelt werden. Besteht eine Leberzirrhose im Child-Pugh Stadium A oder B, oder sind die Patient:innen zur Lebertransplantation gelistet, sollen im Rahmen von HCC-Früherkennungsprogrammen im Abstand von sechs Monaten Ultraschallkontrollen mit Alpha-Fetoprotein (AFP) Bestimmung, einem Tumormarker für Leberkarzinome, erfolgen. Bei Patient:innen mit NAFLD sollte regelmäßig der Fibrosestatus bestimmt werden. Als bildgebende Diagnostik eignet sich das Kontrastmittel-MRT aufgrund der typischen Kontrastmitteldynamik des HCC. Ist diese in zwei

unabhängigen Bildgebungen nicht eindeutig zu interpretieren, sollte, auch bei kurativer Therapieabsicht, eine Leberbiopsie zur definitiven Diagnostik vollzogen werden. Bei Patient:innen ohne Leberzirrhose vor definitiver Therapie und in palliativen Situationen ist immer eine Biopsie anzustreben [36]. Das aktuell von der European Association for the Study of the Liver (EASL) präferierte Staging-System ist die Barcelona Clinic Liver Cancer (BCLC) Klassifikation, anhand der Patient:innen basierend auf Tumorstadium, Allgemeinzustand und Leberfunktion, mittels Child-Pugh Score, in vier Gruppen eingeteilt werden [37]. Der Child-Pugh Score ist ein etabliertes System, mit dessen Hilfe die Leberfunktion abgeschätzt wird [38]. Zu den gemessenen Parameter zählen Albumin- und Billirubinkonzentration im Serum, der Quick-Wert (Gerinnungsparameter), sowie das Ausmaß an Aszitis und hepatischer Enzephalopathie.

1.2.2 Therapie

Die leitliniengerechte Therapie nach AWMF richtet sich nach Tumorgröße, Befallsmuster und erhaltener Leberfunktion, sowie der portalen Hypertension bei Leberzirrhose [36]. Die Lebertransplantation sichert Patient:innen mit lokal begrenzten Befunden (< 5 cm) ohne makrovaskuläre Gefäßinvasionen (Mailand-Kriterien) die besten 5-Jahres-Überlebensraten und sollte als Therapieoption in interdisziplinären Tumorkonferenzen diskutiert werden. Sind die Kriterien für eine Transplantation nicht erfüllt, stehen mit der Resektion, der perkutanen Thermoablation und der transarteriellen Chemoembolisation (TACE) (+Thermoablation) als interventionelle Therapien, Möglichkeiten zum Downstaging der Karzinome bis zum Erfüllen der Kriterien, bzw. Bridging zum Aufrechterhalten der Kriterien bis zum OP-Termin, weitere Therapieoptionen zur Verfügung. Ist eine Transplantation nicht möglich, sind Resektion und Thermoablation zwei potenziell kurative lokaltherapeutische Behandlungsmethoden. Zu beachten ist, dass eine Resektion ab einer Tumorgröße von > 3 cm der Thermoablation zu bevorzugen ist. Hingegen sollte die Thermoablation Patient:innen mit kleinen Tumoren bei schlechter Leberfunktion angeboten werden. Ist kein kurativer Ansatz möglich, bietet die TACE, jedoch nur bei erhaltener Leberfunktion, eine Therapieoption [36].

Treten Fernmetastasen auf oder ist der Lokalbefund nicht kontrollierbar, bzw. resezierbar, muss zur systemischen Medikamentengabe übergegangen werden (Abbildung 3). Erstlinientherapie bei erhaltener Leberfunktion (Child-Pugh-Stadium A) ist die kombinierte Gabe aus den Antikörpern gegen den programmed death-ligand 1 (PD-L1) mit Atezolizumab und den Vascular Endothelial Growth Factor (VEGF) mit Bevacizumab. Bei

Kontraindikationen oder Unverträglichkeiten können die Tyrosinkinase-Inhibitoren Sorafenib oder Lenvatinib verabreicht werden. Bei Tumorprogression sollte mit Sorafenib therapiert werden und bei anhaltender Progression unter Sorafenib-Therapie auf Regorafenib oder Cabozantinib (Tyrosinkinase-Inhibitoren), bzw. dem VEGFR2-Antikörper Ramucirumab gewechselt werden (nur bei AFP-Werten ≥ 400 ng/ml) [36].

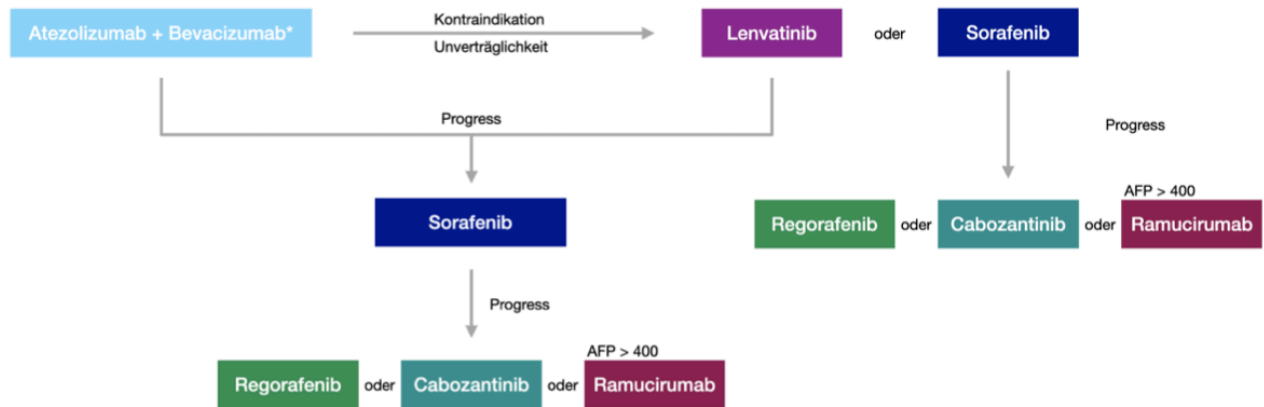


Abbildung 3: Systemische Therapie des hepatozellulären Karzinoms nach aktuellen S3 AWMF-Leitlinien [36].

Patient:innen mit eingeschränkter Leberfunktion (Child-Pugh-Stadium B) kann eine Therapie mit Sorafenib oder dem anti-PD-1-Antikörper (Nivolumab) angeboten werden [36].

Trotz neuer Therapieformen ist das HCC eine meist tödlich verlaufende Erkrankung. Nach kurativer Lebertransplantation liegt die 5-Jahres-Gesamt-Überlebensrate bei 56,2 % [39]. Bei HCCs in fortgeschrittenen Stadien ist mit einer geringen Lebenserwartung zu rechnen. Unter Erstlinientherapie überlebten schätzungsweise nur 67,2 % die ersten 12 Monate nach Studienbeginn [40]. Zur besseren Therapie müssen neue Medikamente erforscht werden, um den Patient:innen längeres Überleben mit besserer Lebensqualität zu gewährleisten.

1.3 Histondeacetylase-Inhibitoren

Als eine neue interessante Substanzklasse, die schon Einfluss in der Therapie anderer Tumorentitäten gefunden hat, wurden in dieser Arbeit verschiedene neu synthetisierte HDAC-Inhibitoren vorgestellt. In der zweiten Originalpublikation werden die Ergebnisse aus Zellkulturversuchen und ersten in vivo Experimenten des neuartigen HDAC-Inhibitors

SF5-SAHA zur Therapie von Prostata- und hepatozellulären Karzinomen vorgestellt. Vorinostat (SAHA) ist 2006 als erster HDAC-Inhibitor zur Therapie des kutanen T-Zell-Lymphoms (CTCL) von der Food and Drug Administration (FDA) zugelassen worden [41]. Weitere Zulassungen zur Therapie von CTCL (Romidepsin), sowie von peripheren T-Zell Lymphomen (PTCL, Belinostat, Romidepsin) und Multiplen Myelomen (MM, Panobinostat) folgten und diverse Substrate werden derzeit zur Therapie in soliden Tumoren und hämatologischen Krebserkrankungen geprüft [42-46].

1.3.1 Eigenschaften und Funktionsweise von Histondeacetylasen

Die Desoxyribonukleinsäure (DNA) eukaryoter Zellen bildet im Nukleosom einen Okta-mer-Komplex, bestehend aus den paarigen Histonen H2A, H2B, H3, H4 und 146 Basen-paaren, die sich 1,65-mal als linksgedrehte Superhelix um den Histonkomplex winden [47, 48]. Schon 1964 wurde erkannt, dass die Acetylierung von Histonen regulierend auf RNA Syntheseprozesse einwirkt [49]. Durch Acetylierung N-terminaler Lysinreste der Histone wird deren positive Ladung neutralisiert, was in einer weniger starken Bindung der negativ geladenen DNS resultiert und dadurch das Ablesen von Transkriptionsfakto-ren ermöglicht (Abbildung 4) [50]. Deacetylierte Histone bewirken hingegen eine enge Bindung der DNA am Histon und verhindern dadurch das Ablesen relevanter DNA-Ab-schnitten wie z. B. Tumorsuppressorgene [45]. Weitere Wirkmechanismen beziehen sich auf post-translationale Modifizierungen der Histone, deren Konformationsänderung durch Effektorproteine mit regulatorischen Funktionen erkannt wird und diese rekrutiert [51]. Die histonmodifizierenden Enzyme, verantwortlich für beschriebene Prozesse, sind Histo-nacetylasen (HATs) und Histondeacetylasen (HDACs). Bisher wurden 18 HDAC-Subty-pen in Säugetieren erforscht, die anhand von Sequenzähnlichkeiten zu Hefeproteinen (*S. cerevisiae*) in vier Klassen eingeteilt werden. Die der Klasse I zugehörigen HDAC 1, 2, 3 und 8 treten ubiquitär im Körper auf und sind hauptsächlich im Zellkern lokalisiert. HDAC 4, 5, 7, und 9 werden der Klasse IIa und HDAC 6 und 10 der Klasse IIb zugeordnet. HDAC 5 und 9 treten vor allem in Muskeln, Herz und Gehirn auf. Wohingegen HDAC 4 vermehrt in Gehirn und Skelett exprimiert wird. HDAC 7 findet sich gehäuft in Endothel-zellen und Thymozyten wieder. Klasse IIa HDACs sind zumindest teilweise auch im Zy-toplasma der Zellen auffindbar, wohingegen Klasse IIb HDACs im extranukleären Raum dominieren [52, 53]. Besonders interessant ist das zytosolische HDAC 6 mit Chaperon modulierender Funktion und Auswirkungen auf das Zytoskelett [54]. Der Klasse III wer-

den die sieben Nicotin-Amid-Adenin-Dinukleotid (NAD⁺) -abhängigen histondeacetylierenden Sirtuine (SIRT 1-7, silent information regulator 2 (Sir2) proteins) zugeordnet, anzutreffen in Zellkern und Zytoplasma (SIRT1 und 2), Zellkern und Mitochondrien (SIRT 3), Mitochondrien (SIRT 4 und 5), Zellkern (SIRT 6) oder Nucleolus (SIRT7). Der HDAC Klasse IV ist ausschließlich HDAC 11 zugeordnet, welches sich in Gehirn, Herz, Muskel, Niere und Hoden anreichert [52, 53].

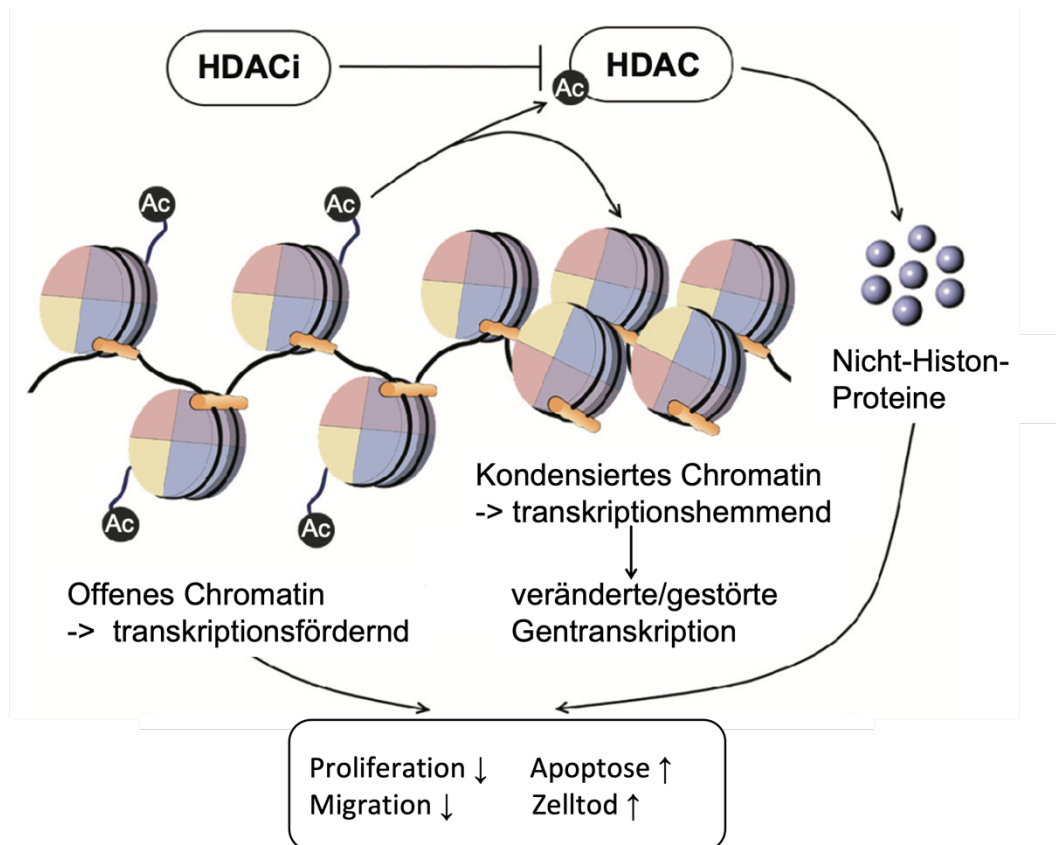


Abbildung 4: Funktionsweise von HDAC-Inhibitoren. Modifiziert aus [55].

1.3.2 Überexpression von Histondeacetylasen in HCC und PCa

Anhand zahlreicher Studien konnte gezeigt werden, dass HDACs in soliden Tumoren, darunter auch PCa und HCC, überexprimiert werden und eine entscheidende Rolle in der Karzinogenese spielen [56-59]. Neben der bereits beschriebenen Funktionsweisen mit direkter Wirkung auf das Chromatin haben HDACs auch indirekten Einfluss auf die Genexpression durch Modifizierung von Nicht-Histon Proteinen wie z. B. DNA-bindende Proteine, Transkriptionsfaktoren, Signaltransduktionsmoleküle, DNA-Reparaturproteine und

Chaperone [55, 60]. Die zahlreichen antineoplastischen Eigenschaften von HDAC-Inhibitoren, zusammengefasst in Abbildung 5, sprechen dafür, dass HDAC-Inhibitoren eine vielversprechende neue Alternative zur Therapie von Karzinomen darstellen [61].

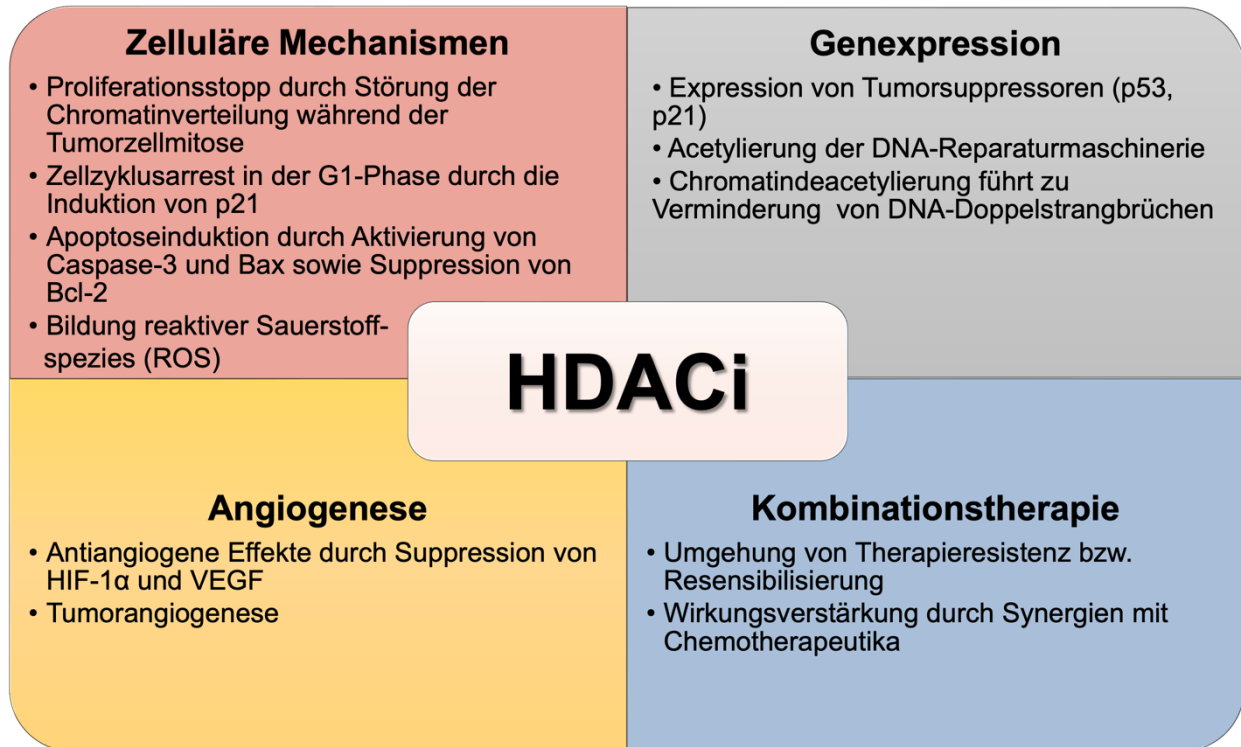


Abbildung 5: Antineoplastische Eigenschaften von HDAC-Inhibitoren in Tumorzellen. Modifiziert aus [62].

1.3.3 Chimäre HDAC-Inhibitor basierte Substanzen

In den letzten Jahren rückten immer mehr Substanzen in den Fokus, die pharmakologische Eigenschaften zweier Moleküle in sich vereinen und die daher als chimäre Inhibitoren (abgeleitet von Chimaera = Mischwesen der griechischen Mythologie; synonym: duale Inhibitoren/Dualinhibitoren) bezeichnet werden [63]. Durch eine gleichzeitige „Bekämpfung“ des Tumors an mehreren zellulären Zielstrukturen erhofft man sich eine therapeutisch wirksamere Antwort mit geringerer Toxizität [64]. Des Weiteren verspricht man sich eine geringere Therapieresistenz der Tumoren, gerade in der Kombination mit HDAC-Inhibitoren, da diese Resistenzmechanismen wie „Gen-silencing“ entgegenwirken [65, 66]. Als Kombinationspartner bieten sich Kinase-Inhibitoren an, die als „Capping-Gruppe“ (rot) ohne Funktionsverlust über einen Linker (gelb) an die inhibitorische Zinkbindungsgruppe (Hydroxamsäurerest, blau) von HDAC-Inhibitoren, gekoppelt werden können (Abbildung 6) [62]. In der ersten Originalpublikation zu 3BrQuin-SAHA und 3Cl-

Quin SAHA werden zwei neu synthetisierte chimäre EGFR/HDAC-Inhibitoren vorgestellt. Die EGFR-Tyrosinkinase-inhibitorischen Pharmakophore der neuen Dualinhibitoren sind neu synthetisierte Tyrphostin- und Indazole-Derivate. Die EGFR-inhibierende Wirkung dieser Verbindungen ist seit längerem bekannt und mit Gefitinib, Erlotinib und Lapatinib existieren zugelassene „small molecule“ Medikamente, die durch Inhibition des Epidermal Growth Factor Receptor (EGFR) und dessen Tyrosinkinase-Aktivität ihre antikanzerogene Wirkung entfalten [67-69]. Die Überexpression des EGFRs ist außerdem in sowohl PCa als auch HCC nachgewiesen und geht mit einer schlechten Tumorprognose einher [70-72]. Aus der Kombination mit einem HDAC-Inhibitor erhofft man sich eine synergistisch wirkende Kombination aus Substanzen gefunden zu haben, die außerdem Resistenzentwicklungen entgegenwirken kann [62].

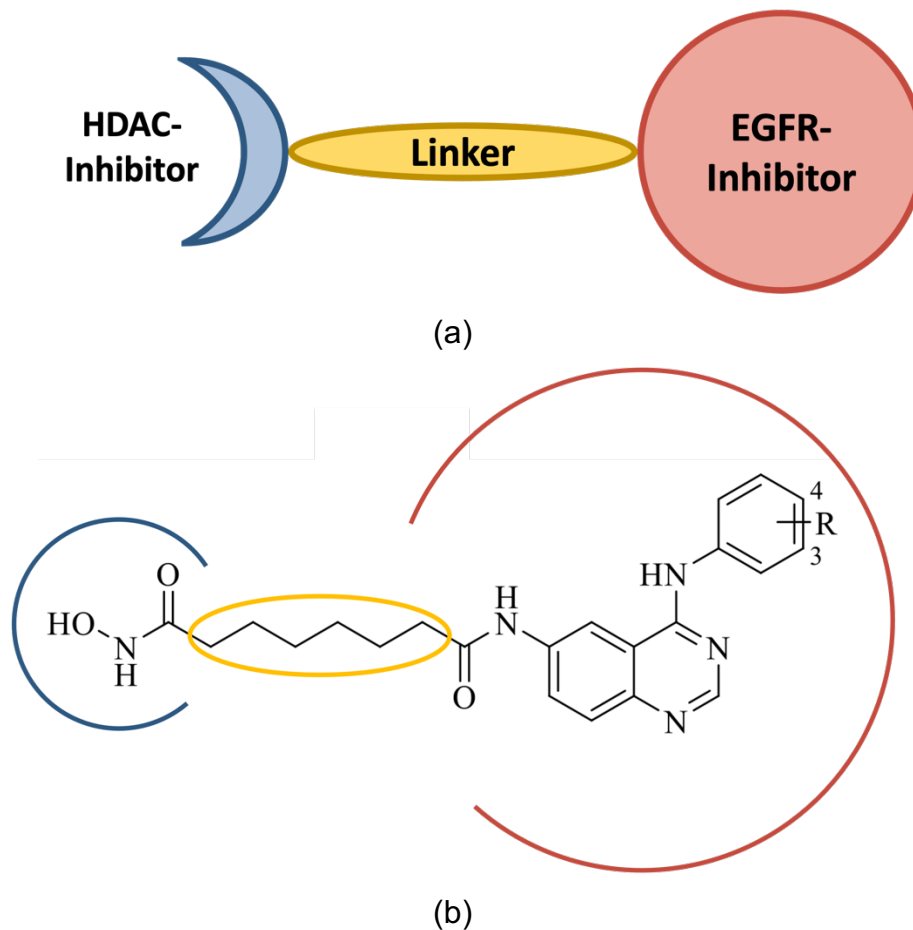


Abbildung 6: Allgemeine Darstellung von chimären EGFR/HDAC-Inhibitoren. (a) Abgebildet als schematische Zeichnung und (b) als Strukturformel für 3BrQuin-SAHA und 3CIQuin-SAHA (R = 3-Br bzw. 3-Cl). Modifiziert aus [63, 73].

Aus der Besonderheit der dualen Inhibitoren, dass nur ein Pharmakon, anders als bei Kombinationstherapie aus zwei oder mehreren Medikamenten, verabreicht wird, erhofft

man sich unterschiedliche Vorteile. Die Gabe einer Substanz, anstatt von zwei Einzelpräparaten, mindert die Wahrscheinlichkeit von Arzneimittelwechselwirkungen, reduziert das Auftreten von unerwünschten Nebenwirkungen und die besser verständliche Pharmakokinetik ermöglicht es, Dosierungen leichter anzupassen [63, 64, 74]. Dadurch, dass nur ein Molekül von den Zellen der unterschiedlichen Gewebe aufgenommen wird, kann garantiert werden, dass eine gleichmäßige Verteilung der beiden Pharmakophoren des chimären Inhibitors in den Zielstrukturen vorliegt und sich diese nicht zu unterschiedlichen Anteilen im Körper verteilen. Durch die Verabreichung von nur einem Medikament anstelle mehrerer, erhofft man sich außerdem eine bessere Compliance der Patient:innen [64, 74]. Auch bei der Herstellung von Medikamenten muss nicht mehr auf unterschiedliche Löslichkeiten der Einzelsubstanzen, die zur Ausfällung führen könnten und die Aufnahme erschweren, geachtet werden [63, 64]. Ein weiteres nicht zu unterschätzendes Argument ist die Wirtschaftlichkeit, da bei neu entwickelten Einzelsubstanzen, diese normalerweise erst separat auf ihre Sicherheit geprüft werden, bevor eine Kombination aus ihnen getestet werden kann. Besonders, wenn die Kombinationspräparate von unterschiedlichen Pharmaunternehmen hergestellt worden sind und Eigentumsrechte zu klären sind, kann dies zu Verzögerungen und Mehrkosten führen [64, 74]. In vitro Arbeiten konnten zeigen, dass die Gabe von chimären Inhibitoren eine stärker inhibierende Antwort auf das Zellwachstum als die der Kombination aus den beiden „Mutter-Substanzen“ hervorruft [75-77].

1.4 Zielsetzung

Ziel dieser Arbeit war es, sich aus präklinischer Sicht mit neuen Therapieformen des Prostata- und hepatozellulären Karzinomen auseinanderzusetzen. Ich habe mich mit der Frage beschäftigt, ob neu synthetisierte HDAC-basierte Inhibitoren als eine Option zur Behandlung von soliden Tumoren, wie den oben genannten, geeignet sein könnten. Es wurde der modifizierte HDAC-Inhibitor SF5-SAHA, sowie die dualen EGFR/HDAC-Inhibitoren 3BrQuin-SAHA und 3CIQuin-SAHA, hinsichtlich ihrer Wirkung und spezifischen Wirkmechanismen an humanen PCa- und HCC-Zellmodellen mittels in vitro und in vivo/ovo Untersuchungen charakterisiert.

2. Methodik

2.1 Substanzen

Die in dieser Arbeit untersuchten HDAC inhibierenden Substanzen wurden von unserem Kooperationspartner der Universität Bayreuth (Inst. f. Organische Chemie, Prof. Schobert, Dr. Biersack) bereitgestellt. Eine präzise Beschreibung des Syntheseprozesses der neuartigen dualen Inhibitoren, sowie der monoaktiven Muttersubstanzen, von denen sie sich ableiten, ist der Literatur zu entnehmen [73, 78].

Die verwendeten Referenzsubstanzen Gefitinib (SML1657) und SAHA (SML0061) wurden von Sigma-Aldrich (Merck KGaA, Darmstadt, Deutschland) bezogen.

Von allen Substanzen wurden 10 mM Stocklösungen in Dimethylsulfoxid (DMSO, Sigma-Aldrich, Merck KGaA, Darmstadt, Deutschland) hergestellt und in Aliquots bei -20 Grad Celsius (°C) weg gefroren. Aus den Stocklösungen wurden durch Verdünnung in Kulturmedium oder in phosphatgepufferter Salzlösung (PBS, Thermo Fisher Scientific, Gibco, Waltham, USA) stets frische Arbeitslösungen in den einzusetzenden Konzentrationen für die jeweiligen Versuchsreihen hergestellt. Die finale Konzentration DMSO in den Arbeitslösungen war in allen Experimenten geringer als 0,25 %. In Vorversuchen konnte gezeigt werden, dass bei einer DMSO-Konzentration von weniger als 0,25 % keine Effekte auf das Wachstumsverhalten und Überleben der Zellkulturen vom Lösungsmittel ausgehen.

2.2 Zellkultur

Es wurden zwei Zelllinien, die humanen, epithelial ähnlich wachsenden, hepatozellulären **Hep-G2** Karzinomzellen (ATCC Nummer: HB-8065) und die humanen, epithelähnlich wachsenden, Prostatakarzinomzellen **DU 145** (ATCC Nummer: HTB-81), unterschiedlicher Entitäten solider Tumoren verwendet. Beide Zelllinien wurden in Roswell Park Memorial Institute 1640 Medium (RPMI, Thermo Fisher Scientific, Gibco, Waltham, USA) angereichert mit 10 % fetalem Kälberserum (FKS, C-C-pro, Oberdorla, Deutschland), 2 mM L-glutamine (Thermo Fisher Scientific, Gibco, Waltham, USA), 100 U/ml Penicillin plus 100 mg/ml Streptomycin (Thermo Fisher Scientific, Gibco, Waltham, USA) bei 37 °C, 5 % Kohlenstoffdioxid (CO₂) und befeuchteter Atmosphäre kultiviert. Alle zwei bis drei Tage wurden sie mit neuem Kulturmedium versorgt und bei > 80 % erreichter Konfluenz passagiert und geteilt. Die adhärent wachsenden Tumorzellen wurden mit PBS gewaschen, mit 0,5%iger Trypsin/Ethylendiamintetraessigsäure (EDTA) Lösung (Bio&SELL

GmbH, Nürnberg, Deutschland) abgelöst, in frischem Kulturmedium suspendiert und 1/5 bzw. 2/5 der Zellen wurden zum Weiterwachsen in eine neue Flasche überführt.

2.3 Zellproliferationsversuche

Um das wachstumshemmende Potenzial der Inhibitoren abzuschätzen und die mittlere inhibitorische Konzentration (IC_{50}) bestimmen zu können, wurden Färbungen mit dem DNA-interkalierenden Farbstoff Kristallviolett durchgeführt (Sigma Aldrich, Merck KGaA, Darmstadt, Deutschland) [79]. 1000 Zellen/Well (DU145) oder 1500 Zellen/Well (Hep-G2) wurden in 96-Well Platten ausgesät und für mindestens 48 h bei 37 °C, 5 % CO_2 und befeuchteter Atmosphäre inkubiert. Anschließend wurden die Zellen mit den zu untersuchenden Inhibitoren in steigender Konzentration (0 - 10 Mikromolar, μM) für bis zu 72 h behandelt. Zur anschließenden Zellquantifizierung wurde das Medium abgesaugt, die Wells wurden mit PBS gespült und die verbleibenden, adhärenen Zellen mittels 1 % Glutaraldehyde-Lösung (Merck KGaA, Darmstadt, Deutschland) für 15 Minuten fixiert und anschließend mit DNA markierender 0,1 % Kristallviolett-Lösung für 30 Minuten inkubiert. Danach wurde der ungebundene Farbstoff mit fließendem Wasser (500ml/Min) für 30 Minuten abgespült. Anschließend wurde das zelluläre DNA-gebundene Kristallviolett mittels Zugabe von 100 μl einer 0,2 %iger Triton X-100 (Sigma Aldrich, Merck KGaA, Darmstadt, Deutschland) pro Well wieder in Lösung gebracht. Da die Farbintensität des Kristallvioletts proportional zur zellulären DNA und damit proportional zur Zellzahl ist, kann durch abschließende Extinktionsmessung bei 570 Nanometer (nm) mittels Enzyme-linked Immunosorbent Assay (ELISA)-Reader (Dynex Technologies, Denkendorf, Deutschland) behandlungsinduzierte Änderungen der Zellzahlen gegenüber den unbehandelten Kontrollen bestimmt und IC_{50} Werte \pm SEM der neuartigen Inhibitoren berechnet werden. Aus den Daten wurden zudem Wachstumskinetiken aus $n \geq 3$ unabhängigen Experimenten mittels Graph Pad Prism erstellt.

2.4 Unspezifische Zytotoxizität

Mittels durchgeführter Laktatdehydrogenase (LDH) Assay wurde ermittelt, ob unspezifische Zytotoxizität der Substanzen anteilig für deren antiproliferative Effekte verantwortlich ist [80]. DU145 und Hep-G2 Zellen wurden in 96-Well Platten ausgesät und nach 48 h bei 37 °C, 5 % CO_2 und befeuchteter Atmosphäre für 3 h bzw. 24 h mit den Inhibitoren

(0 - 10 μM) behandelt. Pro Konzentration wurde 3-fach Bestimmungen durchgeführt. Anschließend wurde die LDH-Freisetzung in den Überstand der Zellkulturen, als Marker einer unspezifischen Zellschädigung, mittels Cytotoxicity Detection Kit PLUS LDH (Roche Diagnostics GmbH, Mannheim, Deutschland), detektiert. Hierzu wurden aus jedem Well 100 μl des Überstands entnommen und auf eine neue 96-Well Platte überführt. Das restliche Medium wurde verworfen und die verbliebenen, adhärenen Zellen wurden für 10 Minuten mit 100 μl 2 % Triton X-100-Medium lysiert. Aus dem im Überstand enthaltenen LDH und dem aus den Zellen durch Lyse freigesetzten LDH kann die Gesamt-LDH-Menge pro Ansatz bestimmt werden. Die im Überstand ermittelte LDH-Menge kann so einfach zur Gesamt-LDH-Menge in Beziehung gesetzt werden, um den prozentualen LDH-Anteil im Überstand der Proben zu ermitteln und zu vergleichen. Nach Hinzugabe von Katalysator- und Farbstofflösung konnten die unterschiedlichen Farbtintensitäten mittels Absorptionsmessung bei 490/630 nm nach 15 Minuten mit ELISA-Reader (Dynex Technologies, Denkendorf, Deutschland) quantifiziert werden. Die LDH-Enzymaktivität verhält sich proportional zur Färbung. Die Messungen wurden in Duplikaten in $n = 3$ unabhängigen Experimenten durchgeführt, und die mittleren prozentualen Veränderungen \pm SEM zu den Kontrollen, mit Graph Pad Prism dargestellt.

2.5 EGFR-Kinase-Aktivität

Um das EGFR inhibitorische Potenzial der chimären Inhibitoren abschätzen zu können, wurde ein zellfreier EGFR-Kinase-Assay von Promega angewandt (#3831, Promega, Madison, WI, USA) und zur besseren Einordnung der Ergebnisse der zum klinischen Gebrauch zugelassene EGFR-Tyrosinkinase-Inhibitor Gefitinib (1 μM) als Positivkontrolle mitgeführt [81, 82]. Das Experiment beruht auf mehreren Schritten:

1. Die EGFR-Kinase wandelt Adenosintriphosphat (ATP) in Adenosindiphosphat (ADP) um, was wiederum je nach inhibitorischem Potenzial der Substanzen unterschiedlich stark unterbunden wird. Hierzu wurden 2 μl Enzym (5 ng/ μl) mit 1 μl 3x Inhibitor (Konzentrationen 1 - 10 μM) für 0,5 h präinkubiert und anschließend mit 2 μl eines 10 μM ultra-purem ATP/Substrat (0,2 $\mu\text{g}/\mu\text{l}$ Konzentration) Mix vermischt und für 1 h inkubiert.
2. Im nächsten Schritt wurden für 40 Minuten 5 μl ADP Glo Reagenz (ADP-Glo kinase assay kit; Promega, Madison, WI, USA) hinzugefügt, um noch verbleibendes ATP zu verbrauchen.

3. Als Letztes wurde das entstandene ADP durch Zugabe von 10 μ l Kinase-Detektionsreagenz wieder in ATP umgewandelt und durch Luciferase/Luciferin-Reaktion ein Leuchtsignal, proportional zur Kinase-Aktivität, erzeugt.

Die Lumineszenz wurde mit einem VarioSkan Flash 40053 Mikroplatten-Luminometer (Thermo Fisher Scientific, Waltham, USA) für 1 s gemessen. Alle Schritte wurden bei Raumtemperatur, in einer 384-Well Platte, in Kinase-Reaktions-Puffer, ausgeführt und die Ansätze in Triplikaten angesetzt. Aus den $n = 3$ unabhängigen Experimenten sind die Mittelwerte \pm SEM der prozentualen Abnahme der EGFR-Tyrosinkinase-Aktivität, im Vergleich zur unbehandelten Kontrolle, deren Aktivität auf 100 % gesetzt wurde, angegeben.

2.6 HDAC-Aktivität

Zur Bestimmung der HDAC inhibitorischen Wirkung der neuartigen Substanzen wurden pan-HDAC-Aktivitäts-Assays (566328 Calbiochem, Merck KGaA, Darmstadt, Deutschland) und subtypspezifische HDAC-Assays für die beiden Subtypen HDAC 2 (50062, BPS Biosciences, San Diego, USA) and HDAC 6 (50076-1, BPS Biosciences, San Diego, USA) durchgeführt. Der klinisch zugelassene pan-HDAC-Inhibitor SAHA sowie Trichostatin A wurden als Vergleichssubstanzen mit bekannter HDAC-Inhibitorischer Wirkung (Positivkontrollen) mitgeführt [41, 83].

Pan-HDAC-Aktivitäts-Assay: Als Quelle für HDAC-Aktivität wurden beigefügte nukleäre HeLa (6 - 9 mg Protein/ml) Zellextrakte verwendet. Zur Evaluierung der inhibitorischen Eigenschaften der Inhibitoren wurde wie folgt vorgegangen:

10 μ l 5x Inhibitor-Lösung, bzw. Positivkontrolle und 15 μ l einer 1:30 HeLa-Kernzellextrakt:Puffer Dilution wurden für 15 Minuten präinkubiert und anschließend weitere 15 Minuten mit 25 μ l Substrat inkubiert (Substratkonzentration 50 μ M). Die HDACs der Kernzellextrakte deacetylieren das Substrat, was durch die Inhibitoren verhindert wird. Im zweiten Teil des Experimentes wurden 50 μ l 1x HDAC-Entwickler, versetzt mit 2 μ M Endkonzentration Trichostatin A, für 15 Minuten hinzugegeben und dabei entstandene Fluoreszenzsignale bei ex/em 380/460 nm mit Varioskan Flash Fluorometer (Thermo Fisher Scientific, Waltham, USA) gemessen. Nur deacetyliertes Substrat wird vom Entwickler erkannt, weshalb sich die Fluoreszenzsignale antiproportional zum HDAC-inhibitorischem Potenzial der Substanzen verhalten. Alle Schritte wurden bei Raumtemperatur und in Assay-Puffer durchgeführt. Die Mittelwerte wurden aus den Duplikaten berechnet und auf die maximale HDAC-Aktivität (100 %) unbehandelter Kontrollen bezogen. Die

prozentuale Abnahme der HDAC-Aktivität wurde als $MW \pm SEM$ aus $n = 3$ unabhängigen Experimenten mit Graph Pad Prism berechnet.

Das Experiment der *subtypspezifischen HDACs 2 und 6* hat den gleichen Funktionsmechanismus. 5 μ l der 10x Inhibitor-Lösung (gelöst in ddH₂O) sowie SAHA und Trichostatin A wurden mit 5 μ l aus einer 200 μ M Substrat 3 Vorverdünnung, 5 μ l 1 mg/ml Bovines Serumalbumin (BSA) und 30 μ l HDAC-Assay-Puffer gemischt. Anschließend wurden 5 μ l einer 1 ng/ μ l HDAC 2-Puffer-Lösung bzw. 5 μ l einer 7 ng/ μ l HDAC 6-Puffer-Lösung den Ansätzen, der schwarzen 96-Well-Platte, hinzugegeben und für 30 Minuten bei 37 °C inkubiert. Die Fluoreszenzreaktion wurde durch Zugabe von 50 μ l HDAC-Entwickler gestartet und nach 15-minütiger Inkubation bei Raumtemperatur, bei einer Anregungswellenlänge von 350 nm und einer Emissionswellenlänge von 460 nm, mit Varioskan Flash Fluorometer (Thermo Fisher Scientific, Waltham, USA) ausgelesen. Berechnung und Darstellung der Ergebnisse erfolgte wie beim pan-HDAC-Aktivitäts-Assay.

2.7 Apoptosespezifische Caspase-3-Aktivität

DU145 (80 000/Well) und Hep-G2 (120 000/Well) Zellen wurden in 6-Well-Platten ausgesät und kurz vor dem Erreichen eines konfluenten Wachstums mit aufsteigenden Inhibitor-Konzentrationen für 6 - 24 Stunden inkubiert. Anschließend wurde das Medium abgesaugt, die Zellen wurden mit PBS gewaschen. Zellen, die sich während der Behandlung oder durch das Spülen von den Wells abgelöst hatten, wurden durch Zentrifugation (4000 Umdrehungen pro Minute (revolutions per minute = Rpm), 5 min, 4 °C, Heraeus Labofuge 400R Function Line, Profcontrol GmbH, Schönwalde-Glien, Deutschland) der Überstände bzw. des Spülmediums pelletiert und in die Wells rückgeführt. Nach 24-stündiger Kältelysierung bei -20 °C wurden die aufgebrochenen Zellen in einem Lysepuffer aufgenommen, zentrifugiert (13000 rpm, 15 min, 4 °C Zentrifuge, Centrifuge 5415 R, Eppendorf, Hamburg, Deutschland) und die im Überstand befindlichen Proteine wurden in neue Reaktionsgefäße überführt und der Proteingehalt der Proben wurde mittels Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) quantifiziert. Die Änderungen der Caspase-3-Aktivität wurden durch die Spaltung des fluorogenen Substrats AC-DEVD-AMC (EMD Millipore, Billerica, MA, USA) gemessen [84]. 100 μ l Ly-sat wurde für 1 Stunde bei 37 °C mit der Substratlösung inkubiert (20 μ g/mL AC-DEVD-AMC, 20 mM HEPES, 10 % Glycerin und 2 mM DTT bei pH 7,5). Bei erhöhter Caspase-

3 Aktivität entsteht ein stärkeres Fluoreszenzsignal, welches mittels Varioskan Flash Fluorometer (Thermo Fisher Scientific, Waltham, USA) bei 360/40 nm Extinktion und 460/10 nm Emission erfasst wurde. Die in Duplikaten erhobenen Rohwerte wurden auf die zugehörige Proteinmenge der Probe normalisiert und in Relation zu einer als 1 (basale Caspase-3-Aktivität) definierten unbehandelten Kontrolle gesetzt. Die Mittelwerte \pm SEM aus $n = 3$ unabhängigen Experimenten wurden mit Graph Pad Prism dargestellt.

2.8 Reaktive Sauerstoffspezies (ROS)

Die Messung zytosolischer ROS-Bildung erfolgte mittels CellROX® Orange (Thermo Fisher Scientific, Waltham, USA) [85]. Das membrangängige, nichtfluoreszierende Reagenz reichert sich im Zytoplasma der Zellen an. Durch ROS-vermittelte Oxidation wird CellROX Orange in stark fluoreszierende Form im orange/rötlichen Fluoreszenzbereich überführt, die bei einem Anregungs-/Emissionsniveau von 545/565 nm detektiert werden kann. Für die Experimente wurden DU145 und Hep-G2 Zellen in 96-Well-Platten ausgesät, inkubiert und CellROX® Orange (1 μ M) bei Beimpfung mit den Inhibitoren (1 - 10 μ M) hinzugefügt. Die Platten wurden bei 37 °C, 5 % CO₂ und befeuchteter Atmosphäre weiter kultiviert und die Bildung von ROS nach 3 h, 6 h, 12 h und 24 h mit dem ZOE™ Fluorescent Cell Imager (Biorad, München, Deutschland) gemessen. Vor den Aufnahmen wurde ein Nullabgleich an den unbehandelten Kontrollen vorgenommen, um Autofluoreszenz der Zellen zu unterdrücken (gain 10, exposure 500 für DU145 bzw. 700 Hep-G2, LED intensity 50, contrast 30). Mit denselben Einstellungen wurden mehrere zufällig ausgewählte zentral liegende Regionen von Interesse aufgenommen und die Intensitäten und das zeitliche Auftreten der Fluoreszenzsignale qualitativ zwischen den unterschiedlichen Substanzen und Konzentrationen ausgewertet. Als Experiment interne Kontrolle wurden unbehandelte Zellen nach 30-minütiger Inkubation mit 1,6 mM Wasserstoffperoxid (H₂O₂) mitgeführt. Die Versuche wurden in $n = 3$ unabhängigen Experimenten reproduziert.

2.9 Scratch Assay

Um Änderungen im Migrationsverhalten von DU145 Zellen nach Behandlung mit den neuartigen Inhibitoren zu untersuchen, wurden sog. „Scratch Assays“ durchgeführt, bei denen mit einer 10 μ l-Pipettenspitze eine feine zellfreie Linie/Kreuz in einen konfluent gewachsenen Zellrasen geritzt wird. Unmittelbar danach werden Bilder der Zellrasenverletzung aufgenommen. Anschließend werden die „gescratchten“ Zellrasen mit steigenden

Inhibitorkonzentrationen (0 - 10 μM) für 24 h inkubiert. Nach 12 und 24 Std. werden die Scratches fotografiert, um zu dokumentieren, wie viele Zellen des Randbereiches in diesen zellfreien Spalt eingewandert sind. Für die Aufnahmen wurde ein EVOS M5000 Mikroskop (Thermo Fisher Scientific, Waltham, USA) benutzt und das Migrationsverhalten unbehandelter Kontrollen wurden mit dem der Inhibitor-behandelten Ansätze qualitativ verglichen. Die Untersuchungen wurden in $n = 3$ unabhängigen Experimenten erhoben und ausgewertet.

2.10 Western Blot Analysen

Zum Nachweis oder Ermittlung des Expressionsstatus verschiedener proliferationsregulatorischer Proteine wurden Western Blot Analysen durchgeführt [86]. Zellen wurden in 100 mm Petrischalen ausgesät, bis zur annähernden Konfluenz gezüchtet und anschließend mit den zu untersuchenden Substanzen, in verschiedenen Konzentrationen (1 - 10 μM) behandelt. SAHA und Gefitinib wurden zur Referenz mitgeführt. Nach 24-stündiger Inkubation bei 37 °C, 5 % CO_2 und befeuchteter Atmosphäre wurden die Zellen gewaschen und zum Aufbrechen bei -20 °C eingefroren. Anschließend wurde sie mit Radioimmunpräzipitationspuffer (RIPA)-Puffer, versetzt mit einer cOmplete™ Mini Protease Inhibitor Tablette/10 ml (Roche Diagnostics GmbH, Mannheim, Deutschland), lysiert und mittels Pierce™ Bicinchoninsäure (BCA)-Protein-Assay-Kit (Thermo Fisher Scientific, Waltham, USA) der Proteingehalt quantifiziert. Um eine spätere gleichmäßige Beladung von 20 μg Protein pro Tasche zu ermöglichen, wurden die einzelnen Proteingehälter pro μl derer einer unbehandelten Kontrolle angepasst. Den Proben wurde Laemmli-Puffer und β -Mercaptoethanol (Biorad, München, Deutschland) beigefügt und anschließend wurden sie für 10 Minuten bei 96 °C denaturiert. Je nach Größe der zu untersuchenden Zielproteine wurden 7,5 %ige oder 12 %ige SDS-Gele (Biorad, München, Deutschland) mit Proteinen beladen und die Elektrophorese, bei einer angelegten Spannung von 90 mV, durchgeführt. Nach erfolgreicher Auftrennung durch die Elektrophorese wurden die Proteine auf aktivierte Polyvinylidendifluorid-Membranen (PVDF) über 90 Minuten bei 100 mV übertragen. Vor Applikation der primären Antikörper bei 4 °C über Nacht, wurden die Membranen 60 Minuten mit 1 % BSA (Sigma Aldrich, Merck KGaA, Darmstadt, Deutschland) blockiert. Folgende Primärantikörper wurden verwendet: acetyliertes Histon H3 (1:1000, ab47915 abcam), EGFR (1:500, sc03 Santa Cruz Bio-technology), Poly-

(ADP-Ribose)-Polymerase (PARP) und gespaltenes PARP (1:1000, 11835238 Roche Diagnostics GmbH, Mannheim, Deutschland), HDAC2 (1:1000, 5113S Cell signaling Technologie, Danvers, USA), HDAC6 (1:1000, 7558S Cell signaling Technologie, Danvers, USA) und B-Aktin (1:2000, A5441 Sigma Aldrich, Merck KGaA, Darmstadt, Deutschland), als „Housekeeping-Protein“, zur Bestimmung des Proteingehalts in der jeweiligen Tasche. „Housekeeping-Proteine“ sind Gene zum Erhalt basaler Zellfunktionen, die keiner regulatorischen Einflüsse unterliegen und gleichermaßen in den Zellproben exprimiert sind. Korrespondierend zum jeweiligen Primärantikörper wurden die beladenen Membranen für 60 Minuten, unter ständiger Rotation, bei Raumtemperatur mit Anti-Maus- oder Anti-Kaninchen-Peroxidase-gekoppelten Anti-IgG-Sekundärantikörpern (1:5000 - 1:10000, Sigma Aldrich, Merck KGaA, Darmstadt, Deutschland) inkubiert, um eine Visualisierung der aufgetrennten Proteinbanden im nächsten Schritt zu ermöglichen. Vor und nach diesem Prozess erfolgte eine dreimalige, 10-minütige Waschung mit 1%iger TBS-Tween-Lösung (Sigma Aldrich, Merck KGaA, Darmstadt, Deutschland). Abschließend wurde die Antikörperbindung mit Clarity oder Clarity Max ECL Western Blotting Substraten (Biorad, München, Deutschland) detektiert und mit den Celvin-S Developer (Biostep, Software SnapAndGo) entwickelt. Aus $n = 3$ unabhängig durchgeführten Western Blot Versuchen wurden die Banden mittels ImageJ analysiert. Hierzu wurden die Grauskala der Proteinbanden dargestellt und deren Fläche unter der Kurve (area under the curve = AUC) bestimmt. Die Werte wurden zum Ausgleich differierender Proteinbeladungen zwischen den Proben auf die B-Aktin-Kontrolle der jeweiligen Bande normalisiert und anschließend mit der unbehandelten Kontrolle des jeweiligen Blots verglichen, die auf 1 gesetzt wurde. Die Mittelwerte \pm SEM wurden mit Graph Pad Prism dargestellt.

2.11 In vivo/ovo Evaluation

Zur Evaluation der antiangiogenetischen Effekte der Inhibitoren wurde die stark vaskularisierte Chorioallantoismembran (CAM) von befruchteten Hühnereiern untersucht [87]. Die fertilisierten Eier wurden in einem Brutkasten bei 60 % Luftfeuchtigkeit und 37,8 °C zur Brut gelagert. Am dritten Tag der Entwicklung wurden die Eier durch ein kleines 2 mm großes Loch an der Oberseite eröffnet, was ein Absinken der CAM auf die Allantois-Höhle bewirkt. Am 10. Entwicklungstag wurde das Loch erweitert, um einen Silikonring (1 cm) auf der CAM platzieren zu können. Nach sechs Stunden wurden 20 μ l 1 - 10 μ M Inhibitor

in Medium oder PBS zur Kontrolle, in den Ring pipettiert und der Effekt auf das Gefäßnetzwerk alle 24 h, für 3 Tage, stereomikroskopisch, mittels Kappa-Digitalkamerasystem (Distelkamp-Electronic, Kaiserslautern, Deutschland), dokumentiert. Die Experimente wurden mit $N \geq 3$ Eiern pro Substanz durchgeführt.

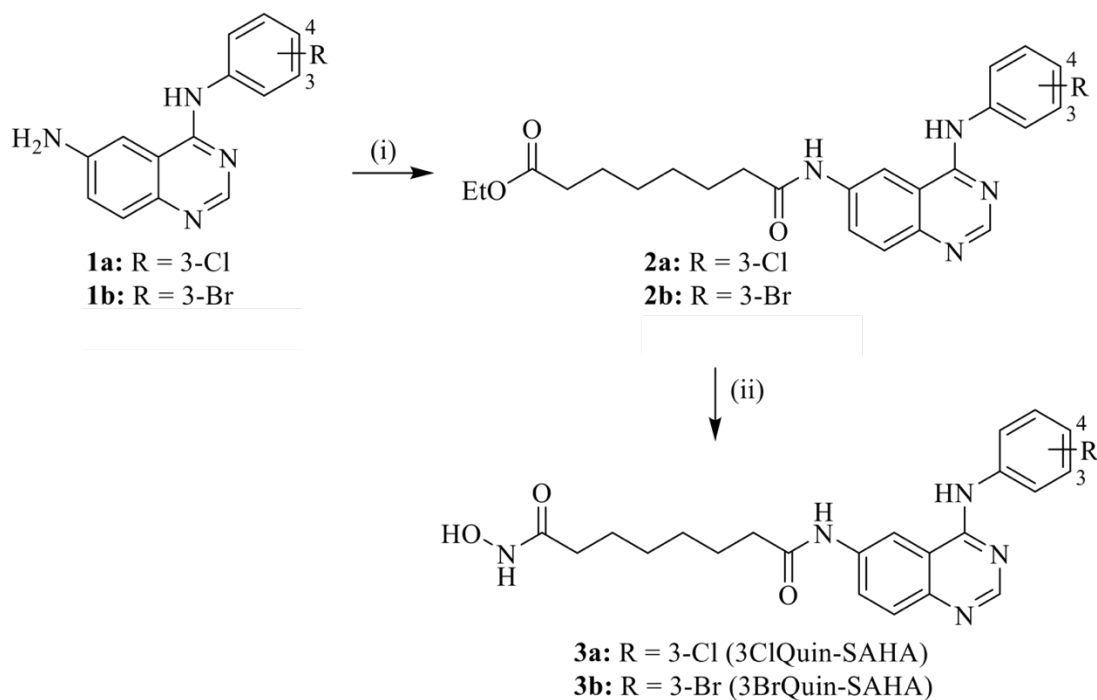
2.12 Statistische Auswertung

Zur statistischen Auswertung wurden 2-wayANOVA Dunnett's post-hoc Tests durchgeführt oder lineare Regressionen gerechnet. Genutzt wurde hierfür GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

3. Ergebnisse

Von unserem Kooperationspartner der Universität Bayreuth (Inst. f. Organische Chemie, Prof. Schobert, Dr. Biersack) wurde mir eine umfangreiche Substanzbibliothek aus neu synthetisierten, HDAC inhibierenden Substanzen zur Verfügung gestellt, deren Wachstums inhibitorische Potenziale in Proliferationsversuchen mittels Kristallviolett färbung untersucht wurde. Diese umfasste sowohl chimäre Inhibitoren, dessen HDAC inhibierende Anteil an einen Tyrosinkinaseinhibitor gegen den EGFR gekoppelt wurde, sowie verbesserte mono HDAC-Inhibitoren. Aus dem Screening hoben sich drei Substanzen als besonders vielversprechend hervor und wurden im Verlauf meines Dissertationsvorhabens genauer untersucht (Abbildung 7). Alle Versuche wurden an zwei Tumorzelllinien unterschiedlicher Entität, DU145 (PCa) und Hep-G2 (HCC), vollzogen.

Die Studien zu den beiden dualen Inhibitoren 3BrQuin-SAHA und 3ClQuin-SAHA wurden in der ersten Originalpublikation veröffentlicht, die zum neuartigen HDAC-Inhibitor SF5-SAHA in der zweiten [73, 78].



(a)

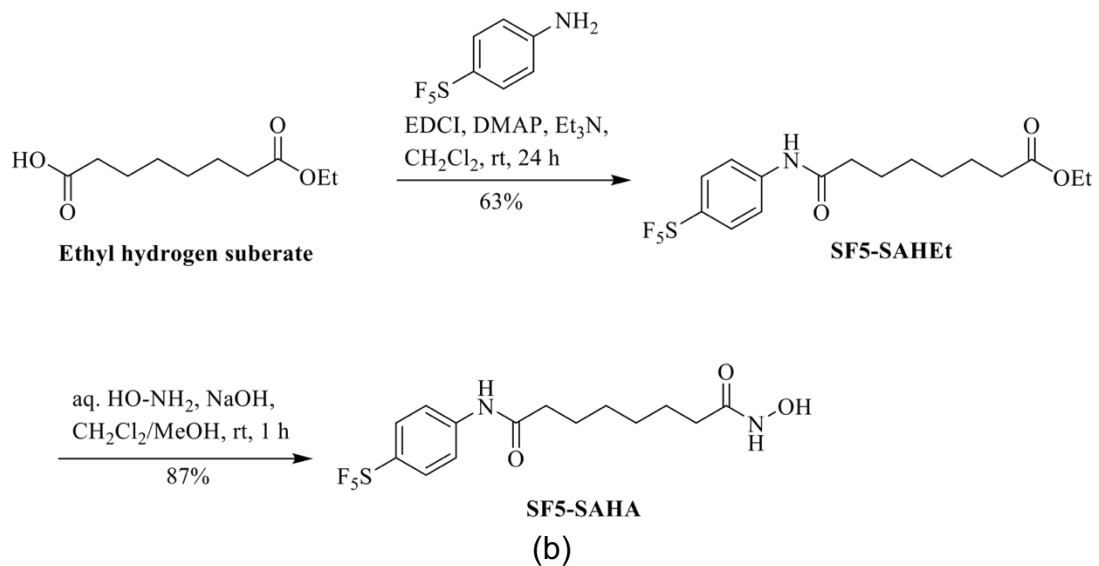


Abbildung 7: Synthese von (a) 3BrQuin-SAHA, 3ClQuin-SAHA und (b) SF5-SAHA. Modifiziert aus [73, 78]

3.1 Zellproliferationsversuche zur Bestimmung des antiproliferativen Potentials

Aus den Daten der Proliferationsversuche konnten die IC_{50} Werte der Inhibitoren für die Zellreihen DU145 und Hep-G2 errechnet werden (Tabelle 3). Die chimären Inhibitoren 3BrQuin-SAHA und 3ClQuin-SAHA haben deutlich prominentere Effekte auf das Wachstum von DU145 und Hep-G2 Zellen als der klinisch relevante mono EGFR Inhibitor Gefitinib und vergleichbar ausgeprägte, mit IC_{50} Werten im niedrigen mikromolaren Bereich, wie der mono HDAC-Inhibitor SAHA. SF5-SAHA, als modifizierter HDAC-Inhibitor, hemmt das Wachstum von DU145 vergleichbar stark in DU145 Zellen und noch fulminanter als SAHA in Hep-G2 Zellen.

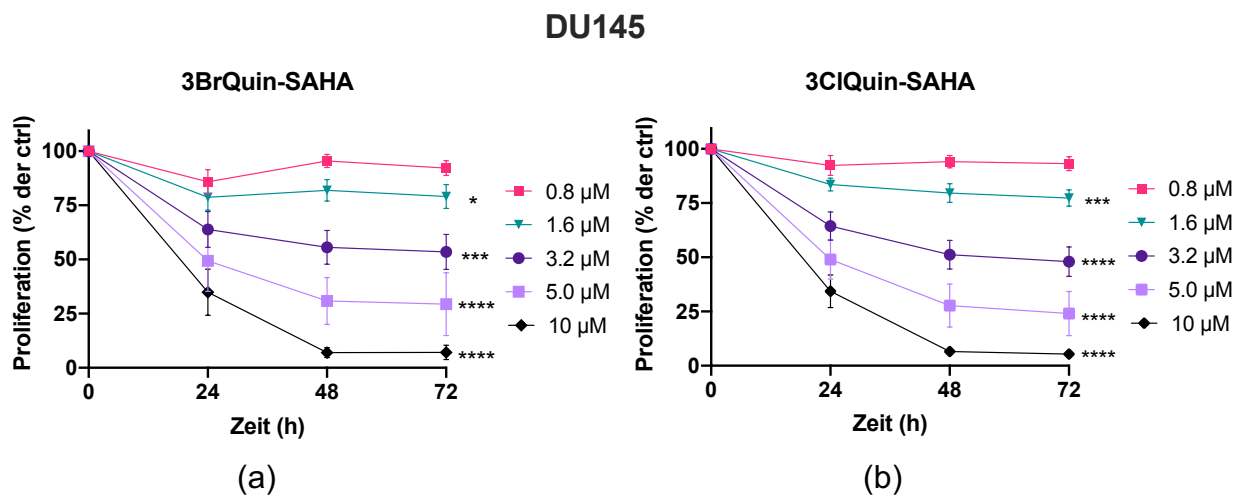
Tabelle 3: Wachstumsinhibition der neuartigen Inhibitoren

Ausgedrückt als IC_{50} Wert (μM) in humanen Prostata- (DU145) und hepatozellulären (Hep-G2) Karzinomzelllinien nach 48 h Behandlung

		Zelllinie	
		DU145	Hep-G2
Inhibitor	3BrQuin-SAHA	3,53 ± 0,23	4,94 ± 0,38
	3CIQuin-SAHA	3,23 ± 0,18	3,92 ± 0,25
	SF5-SAHA	0,73 ± 1,08	1,79 ± 1,06
	SAHA	0,68 ± 0,04	3,22 ± 0,44
	Gefitinib	11,88 ± 2,13	18,53 ± 1,78

SAHA und Gefitinib wurden als klinisch relevante Referenzen für monomodale HDAC- bzw. EGFR-Hemmung verwendet. Alle Ergebnisse sind als Mittelwerte ± SEM aus $n \geq 3$ unabhängigen Experimenten angegeben. Modifiziert aus [73, 78].

Zum besseren Verständnis der Wachstumsdynamiken wurden die antiproliferativen Effekte der Inhibitoren dosisabhängig in Relation zur Zeit gesetzt (Abbildung 8). Eine signifikante Inhibition ist für alle Substanzen in beiden Zellreihen bereits nach 24 h zu beobachten, mit maximalen Zellreduktionen von >95 % in DU145 Zellen und >80 % in Hep-G2 Zellen nach 72 h Behandlung.



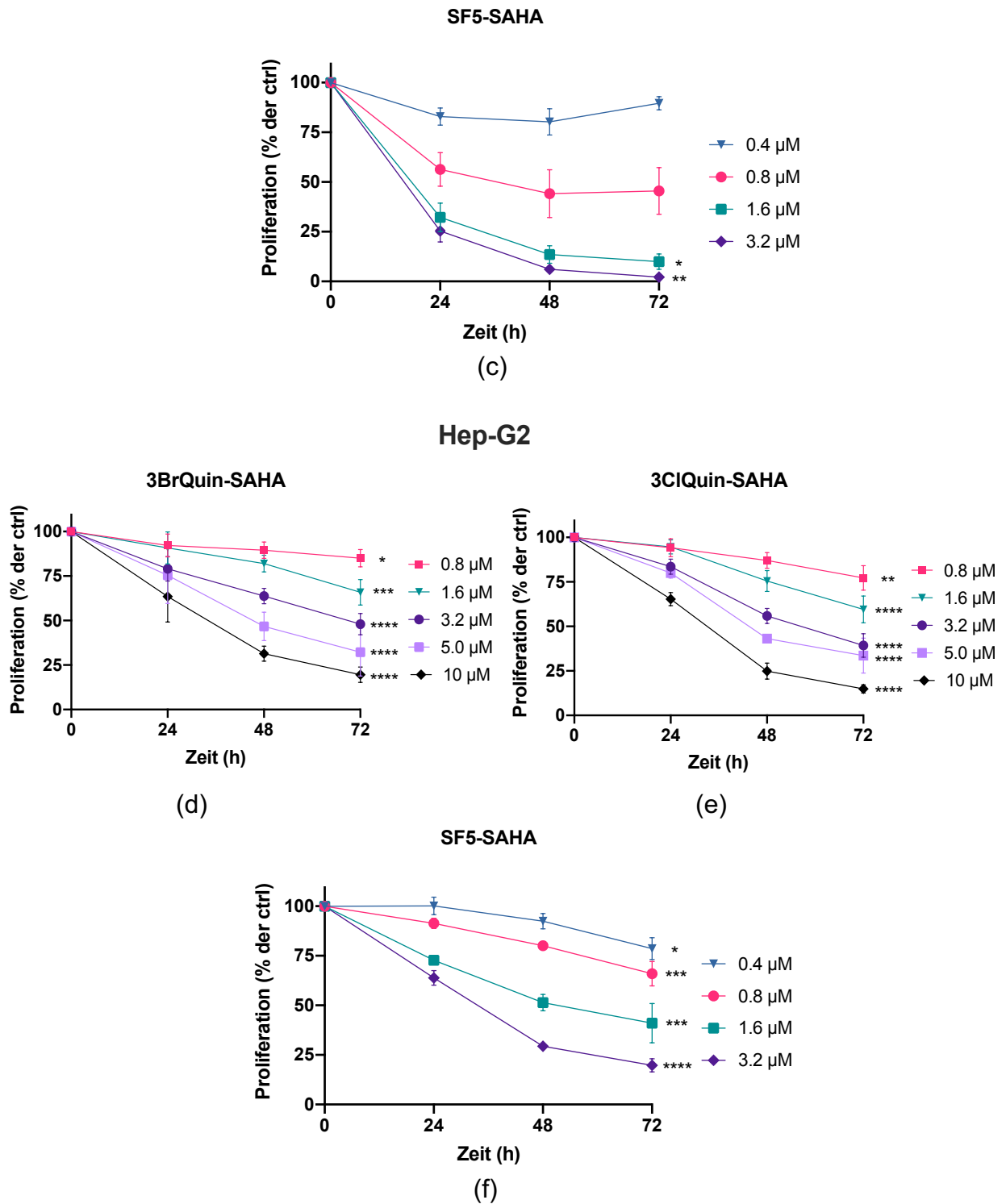
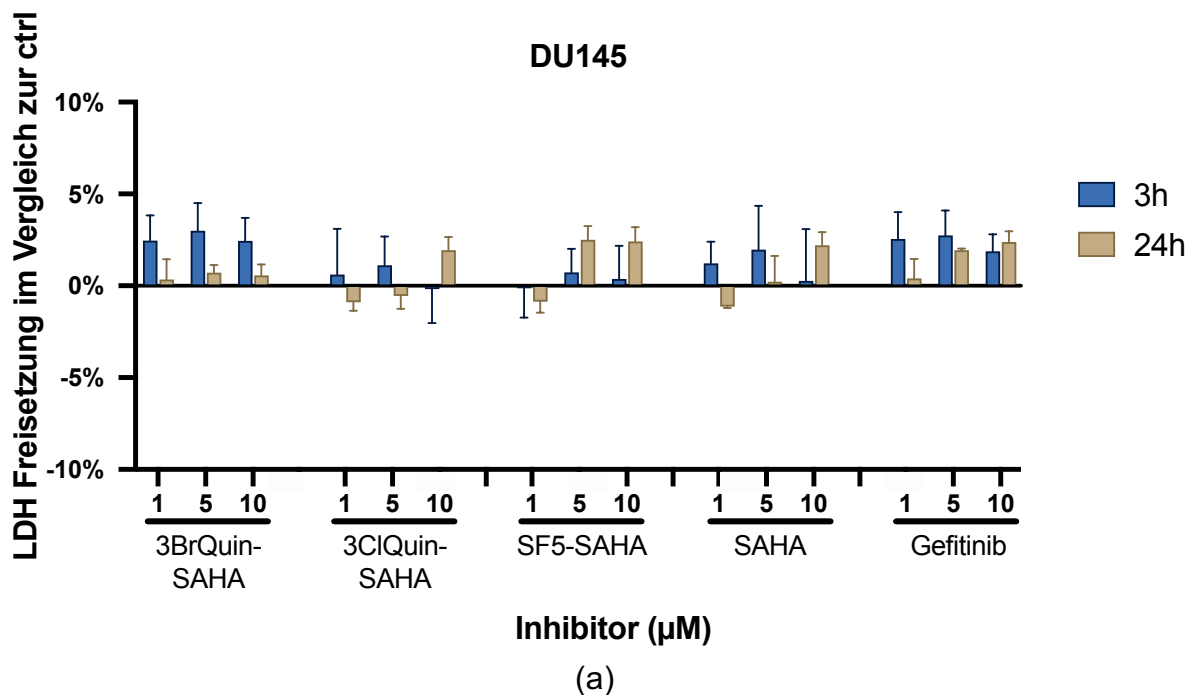


Abbildung 8: Wirkung von (a,d) 3BrQuin-SAHA, (b,e) 3CIQuin-SAHA und (c,f) SF5-SAHA auf (a-c) Pca- und (d-f) HCC-Zellen, dargestellt durch die zeit- und dosisabhängige Wachstumshemmung in Prozent, relativ zu unbehandelten Kontrollzellen, deren Proliferation auf 100 % gesetzt wurde. Die Ergebnisse sind als Mittelwerte \pm SEM von $n \geq 3$ unabhängigen Experimenten angegeben. * $p \leq 0,05$, ** $p \leq 0,005$, *** $p \leq 0,0005$, **** $p \leq 0,0001$; lineare Regression. Modifiziert aus [73, 78].

3.2 Charakterisierung der Wirkweise

3.2.1 Unspezifische Zytotoxizität

Zur weiteren Charakterisierung der zugrundeliegenden Wirkweise wurde zunächst überprüft, ob ggf. die Induktion unspezifischer Zytotoxizität durch die Substanzen einen Anteil an den beobachteten antiproliferativen Effekten hatte. Dies konnte durch sog. Laktatdehydrogenase-Assays ausgeschlossen werden, da im Vergleich zu den unbehandelten Kontrollen keine erhöhte LDH-Freisetzung in den Überstand der Zellkulturen als Marker einer unspezifischen Zellschädigung festgestellt werden konnte (Abbildung 9). Es konnte gezeigt werden, dass weder 3 h nach Behandlungsbeginn noch nach 24 h eine unspezifische Zytotoxizität vorliegt. Die gemessenen Werte oszillieren insignifikant um den Wert der LDH-Freisetzung unter Kontrollbedingungen. Folglich hat die unspezifische Zytotoxizität keinen relevanten Einfluss auf das Wachstumsverhalten von PCa- und HCC-Zellen unter Behandlung der neuen Inhibitoren. Gleiches gilt für die Referenzsubstanzen Gefitinib und SAHA.



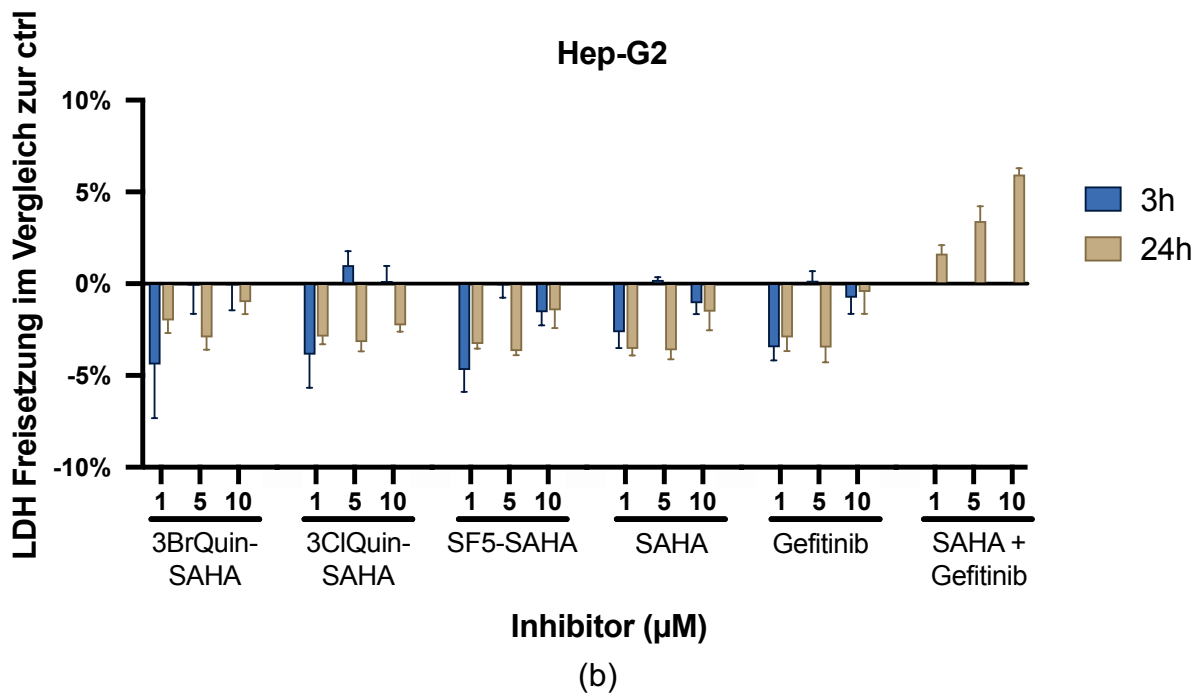


Abbildung 9: Unspezifische zytotoxische Wirkungen von 3BrQuin-SAHA, 3CIQuin-SAHA und SF5-SAHA in (a) Prostata- (DU145) und (b) hepatozellären (Hep-G2) Karzinomzelllinien abgebildet durch die LDH-Freisetzung im Vergleich zu unbehandelten Kontrollen. LDH-Messungen wurde nach 3- und 24-stündiger Inkubation mit 1, 5 oder 10 µM 3BrQuin-SAHA, 3CI-Quin-SAHA, SF5-SAHA sowie SAHA und Gefitinib allein oder in Kombination für die Versuche mit Hep-G2-Zellen, durchgeführt. Die Ergebnisse sind als prozentuale Veränderungen im Vergleich zur basalen LDH-Freisetzung der Kontrollen angegeben. Mittelwerte \pm SEM aus $n = 3$ unabhängigen Experimenten. Modifiziert aus [73, 78].

3.2.2 Spezifische EGFR- und HDAC-Kinase-Aktivität

Ob die dualen Substanzen 3BrQuin-SAHA und 3CIQuin-SAHA wie angenommen in Signalkaskaden des EGFR eingreifen und somit Wachstum regulieren, wurde in einem Lumineszenz-basiertem, zellfreiem EGFR-Kinase-Aktivitäts-Assay überprüft und mit Gefitinib verglichen (Abbildung 10a). Es konnten stark signifikante, dosisabhängige Reduktionen um bis zu ~75 % gemessen werden.

Gleiches wurde für den HDAC inhibitorischen Anteil der chimären Moleküle und SF5-SAHA mittels zellfreiem pan-HDAC Assay vollzogen. Nukleäre HeLa Zellextrakte dienten als Quelle für HDACs, deren Aktivität dosisabhängig bei errechneten IC_{50} Wert von 302 Nanomolar (nM, 3BrQuin-SAHA), 474 nM (3CIQuin-SAHA) und 223 nM (SF5-SAHA)

stark herabgesetzt wurde (Abbildung 10b). Selbiges trifft in noch ausgeprägterer Art für den klinisch relevantesten HDAC-Inhibitor SAHA zu ($IC_{50} = 20 \text{ nm}$).

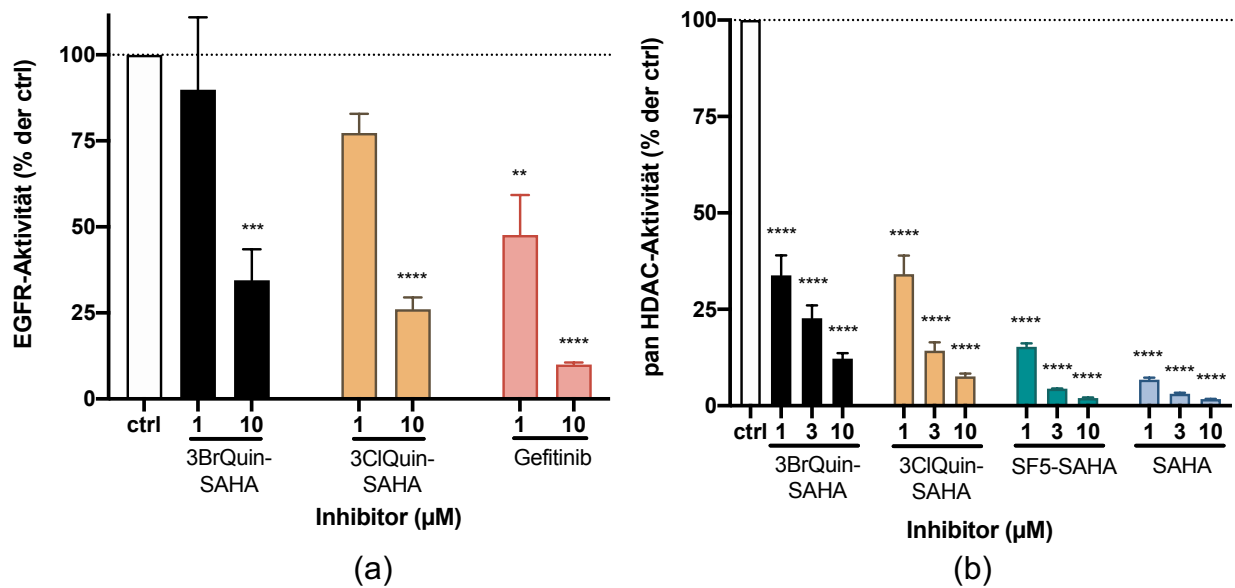


Abbildung 10: Spezifische antiproliferative Wirkung der neuartigen Inhibitoren gezeigt im zellfreien Kinaseassay. (a) Gezeigt wird die dosisabhängige Hemmung der EGFR-Tyrosinkinase-Aktivität durch 3BrQuin-SAHA, 3CIQuin-SAHA und Gefitinib. (b) 3BrQuin-SAHA, 3CIQuin-SAHA, SF5-SAHA und SAHA hemmen dosisabhängig die deacetylierung von Histonen, die aus HeLa-Kernzellextrakten gewonnen wurden. Gemessen wurde der lumineszenzmarkierte Acetylierungszustand der Histone. Die Ergebnisse wurden ins Verhältnis zu Kontrollen gesetzt, die nicht mit den Inhibitoren vorinkubiert wurden und dessen Kinasaktivität bzw. Acetylierungszustand auf 100 % gesetzt wurde. Abgebildet werden die Mittelwerte \pm SEM aus $n = 3$ unabhängigen Experimenten. ** $p \leq 0.005$, *** $p \leq 0.0005$, **** $p \leq 0.0001$; two-way ANOVA Dunnett's post hoc test. Modifiziert aus [73, 78].

3.3 Bestimmung des zellulären Expressionsstatus relevanter Zielstrukturen

Die Ergebnisse aus den zellfreien Versuchen wurden auf Proteinebene mittels Western Blot Studien verifiziert.

Es erfolgte eine Untersuchung des Expressionsniveaus des EGFR in DU145 unter dualer Therapie mit 3BrQuin-SAHA und 3CIQuin-SAHA, sowie unter Therapie mit Gefitinib (Abbildung 11a und c). Interessanterweise wurde der EGFR unter chimärer Therapie deutlich weniger exprimiert als unter Therapie mit Gefitinib. Dies ließ vermuten, dass der HDAC-basierte Teil des chimären Moleküls Einfluss auf das Expressionsverhalten des EGFR in DU145 Zellen hat. In daraufhin durchgeführten Studien zur Expressivität des EGFR unter

SF5-SAHA- und SAHA Behandlung, konnte eine Reduktion des EGFR dargestellt werden.

Die Ergebnisse aus Kinase und Western Blot Untersuchungen lassen einen Synergismus der neuartigen Inhibitoren vermuten, bei dem das Tyrphostin-ähnliche Pharmakophor für eine wirksame Hemmung der EGFR-Tyrosinkinase-Aktivität verantwortlich ist, während das Hydroxamat-Pharmakophor zu einer erheblichen Suppression der Expression des EGFR beiträgt.

Des Weiteren wurden die Effekte auf den Acetylierungsstatus von Histonen unter Therapie mit den neuartigen Inhibitoren auf zellulärer Ebene, in DU145 Zellen untersucht (Abbildung 11b und c). Eine dosisabhängige vermehrte Acetylierung bei steigenden Dosen und folglich mehr Inhibition des Deacetylierungsprozesses, steht im Einklang mit den Ergebnissen aus dem zellfreien pan-Assay.

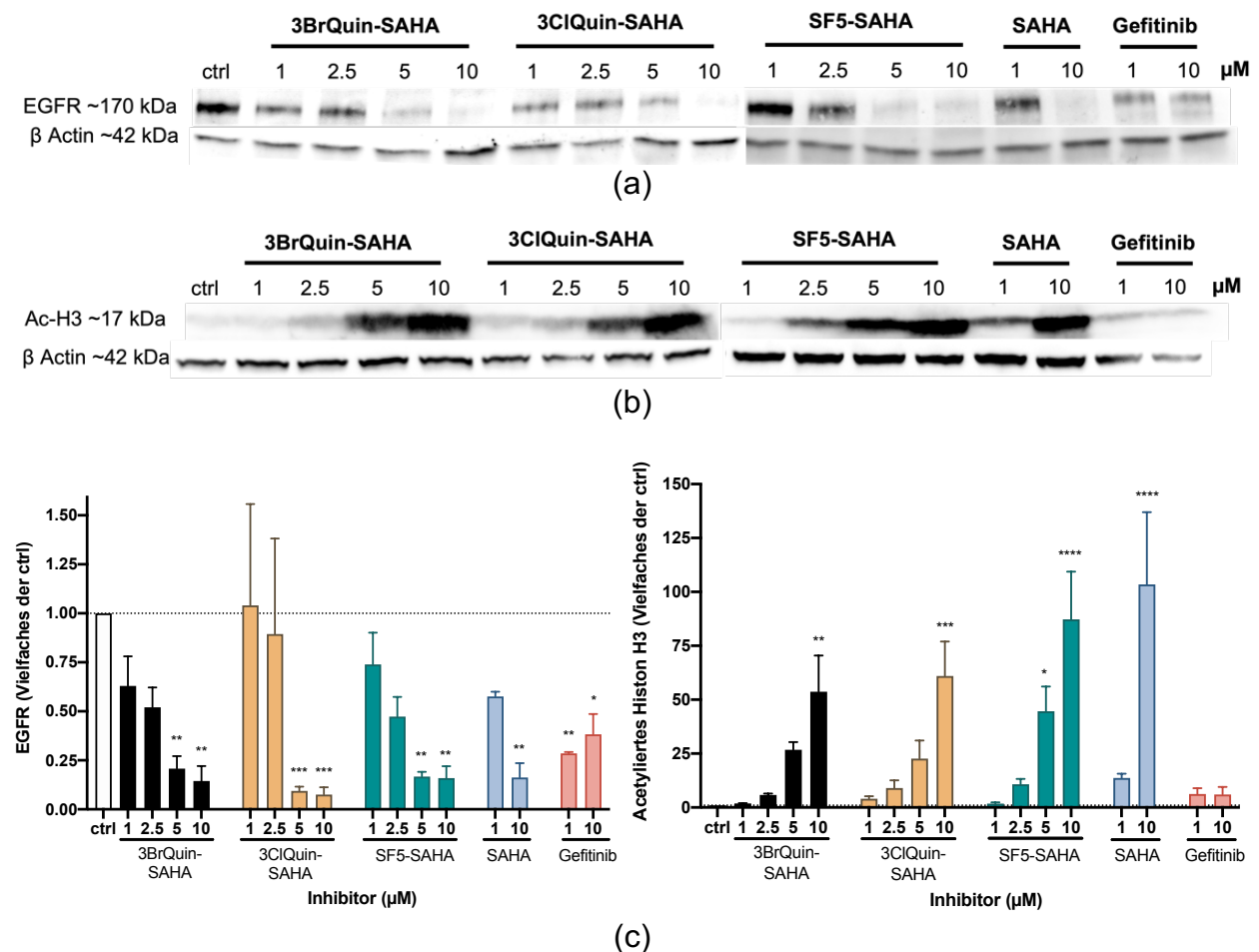


Abbildung 11: (a) Veränderung der EGFR-Expression und (b) der Expression von acetyliertem Histone H3 in DU145-Zellen nach 24-stündiger Inkubation mit 3BrQuin-SAHA, 3CIQuin-SAHA, SF5-SAHA, sowie den Kontrollen SAHA und Gefitinib. β -Aktin wurde als Beladungskontrolle verwendet. Es wird jeweils ein repräsentativer Western Blot aus $n = 3$ Experimenten abgebildet.

(c) Die Grauintensitäten der Banden aus jeweils $n = 3$ Western Blots wurden quantifiziert, an die β -Aktin Beladungskontrolle angepasst und ins Verhältnis zur als 1 gesetzten Kontrolle gesetzt. Die Mittelwerte \pm SEM aus dieser Berechnung wurden grafisch dargestellt. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$, **** $p \leq 0.0001$; two- way ANOVA Dunnett's post hoc test. Modifiziert aus [73, 78].

3.4 Untersuchung der subtypspezifischen HDAC 2 und 6 Inhibition

Zwei HDAC-Subtypen wurden genauer untersucht, nachdem sie mittels Western Blot in unbehandelten DU145 Zellen nachgewiesen werden konnten (Abbildung 12). Die Aktivität von HDAC 6, einem durch seine zytosolische Lokalisation und Chaperon ähnliche Funktion besonders interessanten HDACs, sowie die Aktivität von HDAC 2, konnten unter Therapie mit den neuartigen Substanzen reduziert werden [54].

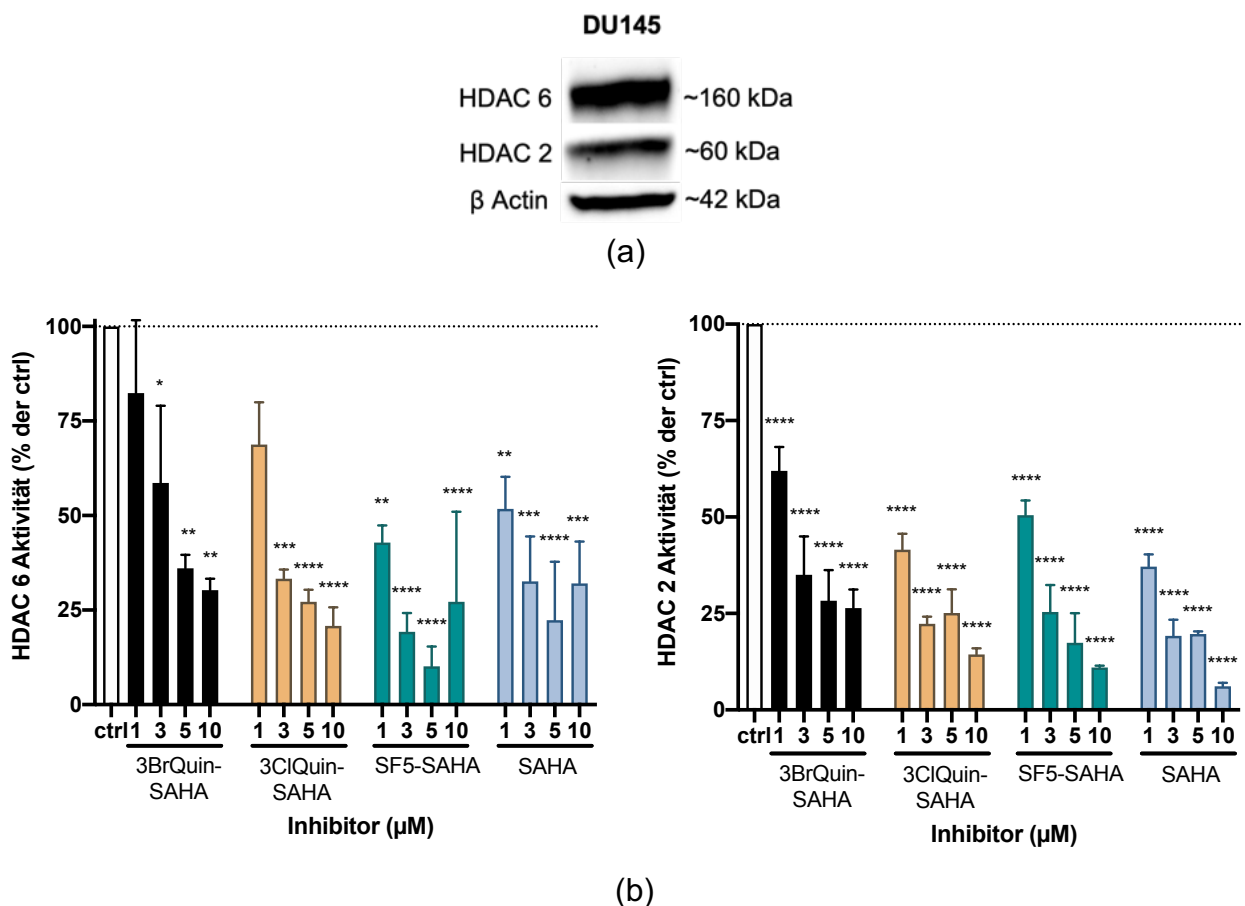
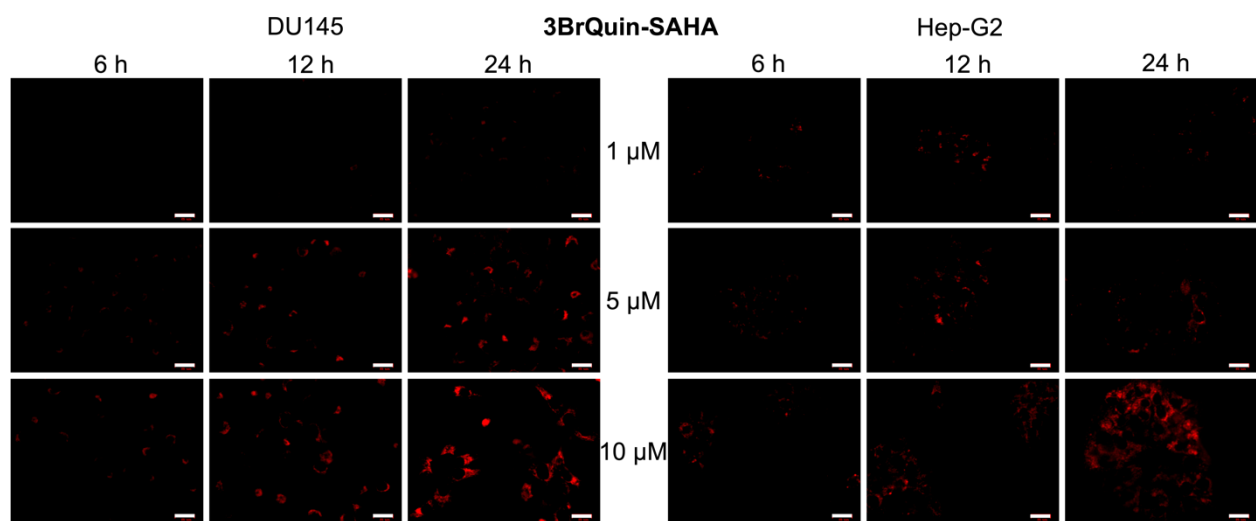


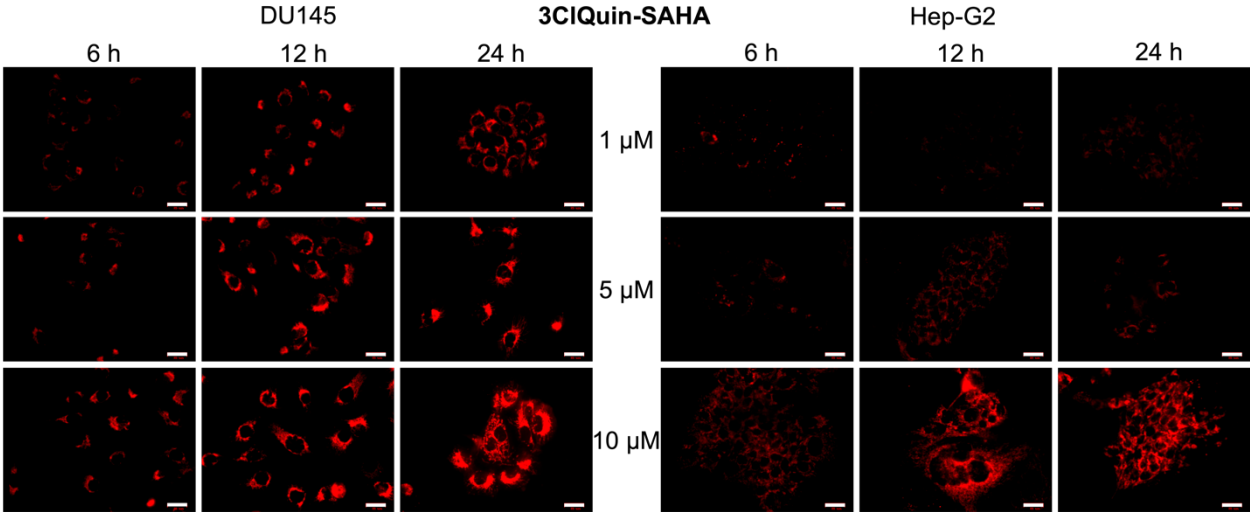
Abbildung 12: HDAC subtypspezifische Hemmung der neuartigen Inhibitoren (a) Western Blot zeigt die Expressionswerte von HDAC 2 und HDAC 6 in unbehandelten DU145-Zellen (b) Dosisabhängige Hemmung der HDAC 6 und 2 Aktivität durch 3BrQuin-SAHA, 3CIQuin-SAHA, SF5-SAHA bzw. SAHA. Die Ergebnisse sind als relative Werte zu den unbehandelten Kontrollen als Mittelwerte \pm SEM aus $n = 3$ unabhängigen Experimenten abgebildet. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$, **** $p \leq 0.0001$; two- way ANOVA Dunnett's post hoc test. Modifiziert aus [73, 78].

3.5 Bildung von freien Sauerstoffradikalen und Induktion von Apoptose

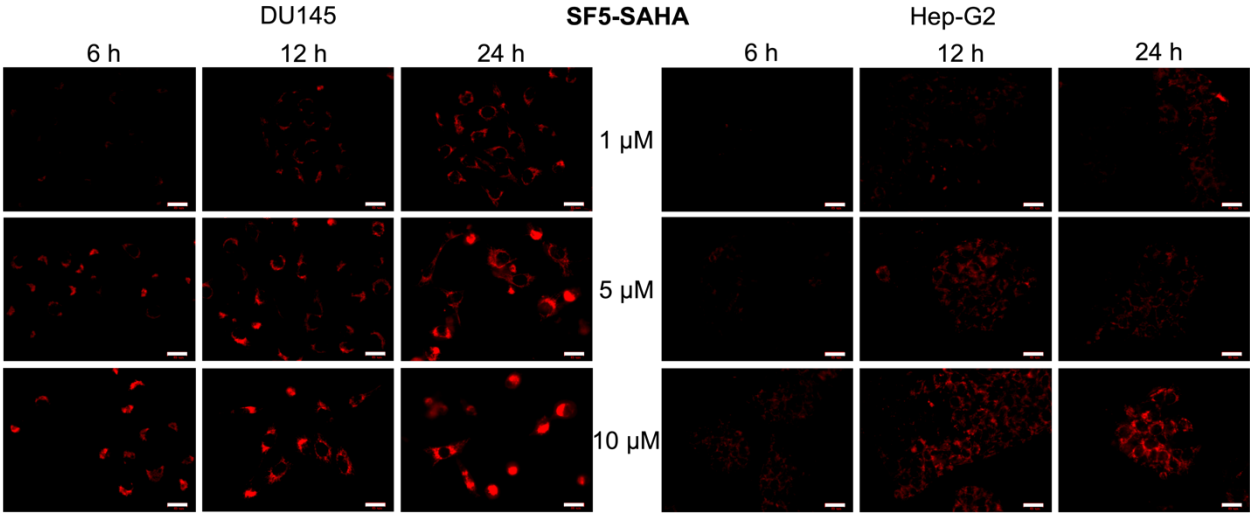
Da bereits beschrieben worden ist, dass HDAC-Inhibitoren die Bildung freier Sauerstoffradikalen (ROS) unterstützen, wurde als Nächstes überprüft, ob die neuartigen Inhibitoren die Bildung dieser im Zytosol von DU145 und Hep-G2 Zellen hervorruft [88]. Anhand des Reagenz CellROX Orange, welches bei Oxidation fluoresziert, wurde nach 3 h, 6 h, 12 h und 24 h nach einer ROS-Induktion geschaut. Nach nur 6 h Inkubation mit 3BrQuin-SAHA, 3CIQuin-SAHA und SF5-SAHA wurde qualitativ ein erhöhtes Fluoreszenzsignal erfasst, dessen Intensität sich nach weiteren 6 h Inkubation nochmals deutlich steigerte und nach 24 h am ausgeprägtesten war (Abbildung 13). Nur unter der Behandlung mit 3CIQuin-SAHA konnte schon nach 6 h in beiden Zelllinien ein eindeutiges Fluoreszenzsignal gemessen werden, welches auch nach 12 h und 24 h die stärkste Intensität, im Vergleich mit den anderen Substanzen bei äquimolaren Konzentrationen, aufweist. Interessant ist, dass selbige Konzentrationen von Gefitinib und SAHA kaum (Gefitinib) bzw. nicht so starke Signale (SAHA) hervorbrachten. Insgesamt wurde in den DU145 Zellen qualitativ ein intensiveres Signal gemessen.



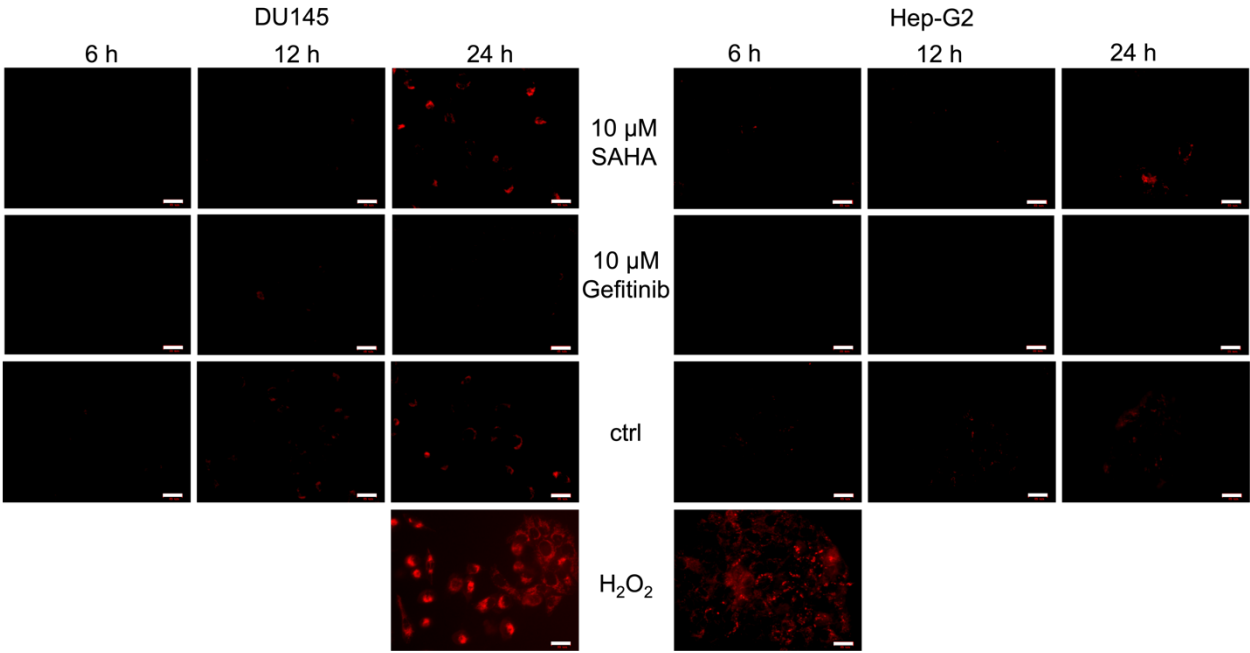
(a)



(b)



(c)

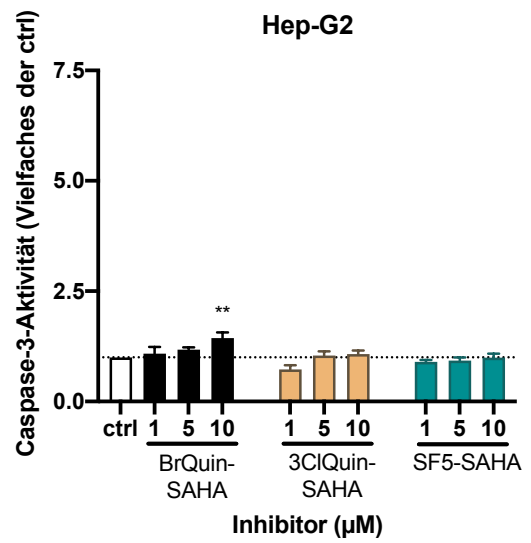
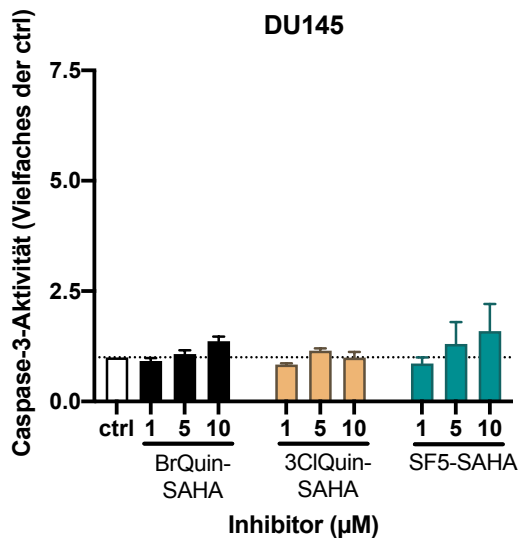


(d)

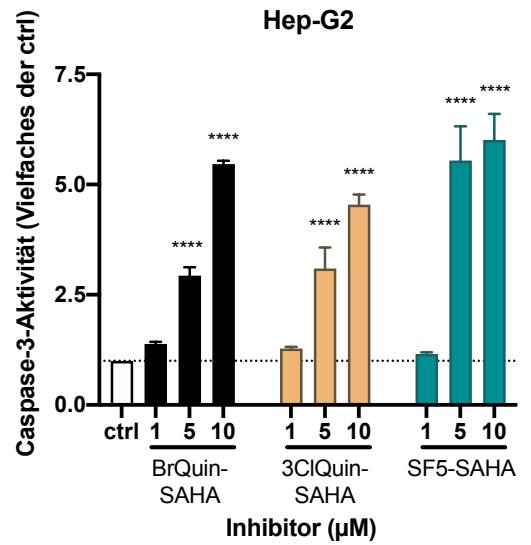
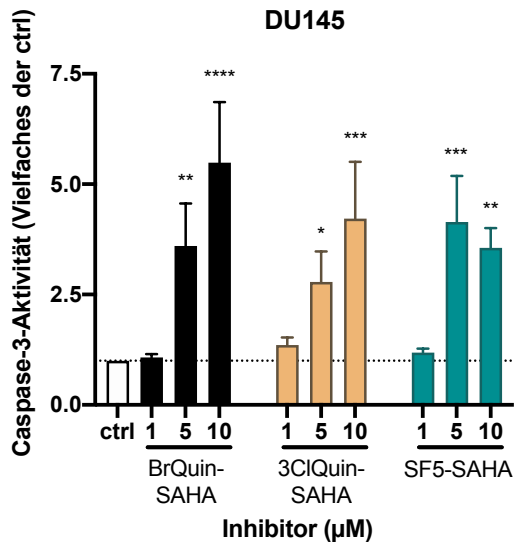
Abbildung 13: Bildung reaktiver Sauerstoffspezies in DU145- und Hep-G2 Zellen durch (a) 3BrQuin-SAHA, (b) 3ClQuin-SAHA und (c) SF5-SAHA, veranschaulicht durch ROS CellROX Orange Färbung. Aufnahmen wurden nach 6-, 12- und 24-stündiger Inkubation mit den Inhibitoren in Konzentrationen 1 - 10 μM gemacht. Unbehandelte Kontrollen, die Referenzsubstanzen (d) SAHA und Gefitinib (10 μM) und Wasserstoffperoxid (H_2O_2) (1,6 mM), als Positivkontrolle, wurden mitgeführt. Der weiße Balken entspricht 25 Mikrometern (μm). Die Abbildungen präsentieren die Ergebnisse aus $n = 3$ unabhängigen Experimenten. Modifiziert aus [73, 78].

Ein weiterer Signalweg, der zum kontrollierten Zelluntergang von Zellen führt, ist die Apoptose, deren Einleitung mit der Entstehung erhöhter ROS Niveaus in Verbindung gebracht wurde [89]. Die Apoptoseaktivität wurde anhand der spezifischen Effektorcaspase-3 in DU145 und Hep-G2 Zellen gemessen (Abbildung 14). 6 h nach Behandlungsbeginn wurde nur unter höchsten Dosen (10 μM) eine erhöhte Caspase-3 Aktivität gegenüber der in unbehandelten Kontrollen gemessen. Anders nach 24 h Behandlung, bei der die Caspase-3-Aktivität um das bis zu 6-Fache gegenüber der Grundaktivität gesteigert wurde, teilweise sogar stärker als durch die Positivkontrolle SAHA. SAHA steigert die Caspase-3-Aktivität nach 24 h im Durchschnitt um das 2,8-, (1 μM), 2,7- (5 μM) und 3,2-fache (10 μM) in DU145 Zellen und um das 1,4-, (1 μM), 5,1- (5 μM) und 5,8-fache (10 μM) in Hep-G2 Zellen. Keine Induktion wurde hingegen unter Behandlung mit äquimolaren Konzentrationen von Gefitinib gemessen (Daten wurden nicht abgebildet). 48 h nach Applikation der Inhibitoren war immer noch eine deutliche, aber schwächere Aktivität messbar. Die Dosis- und Zeitabhängigkeit steht dabei in Einklang mit den Messungen zur Induktion von ROS, wenn die Bildung dieser als ein Initiator für Apoptose gesehen wird.

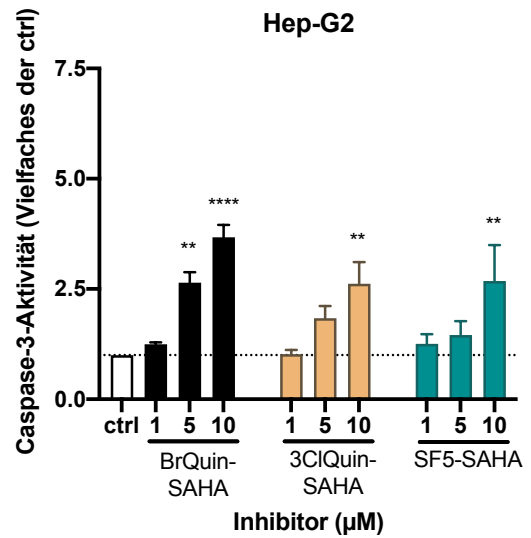
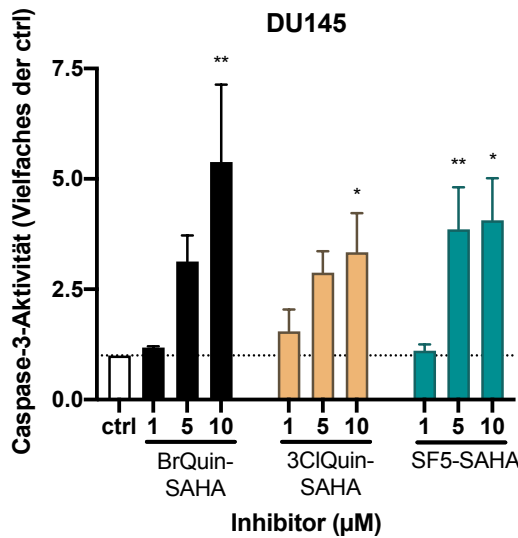
Caspase-3-Induktion nach 6 h



Caspase-3-Induktion nach 24 h



Caspase-3-Induktion nach 48 h

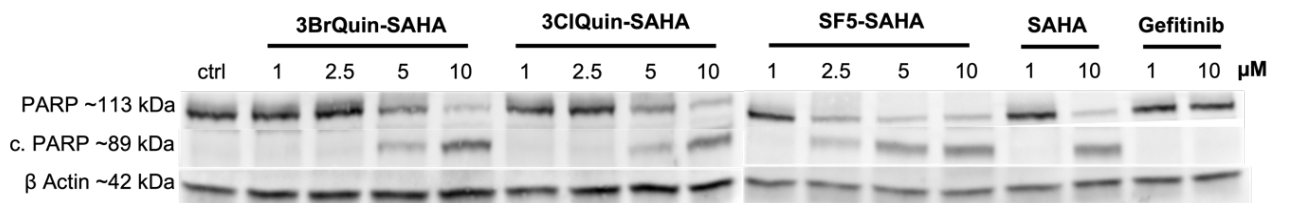


(a)

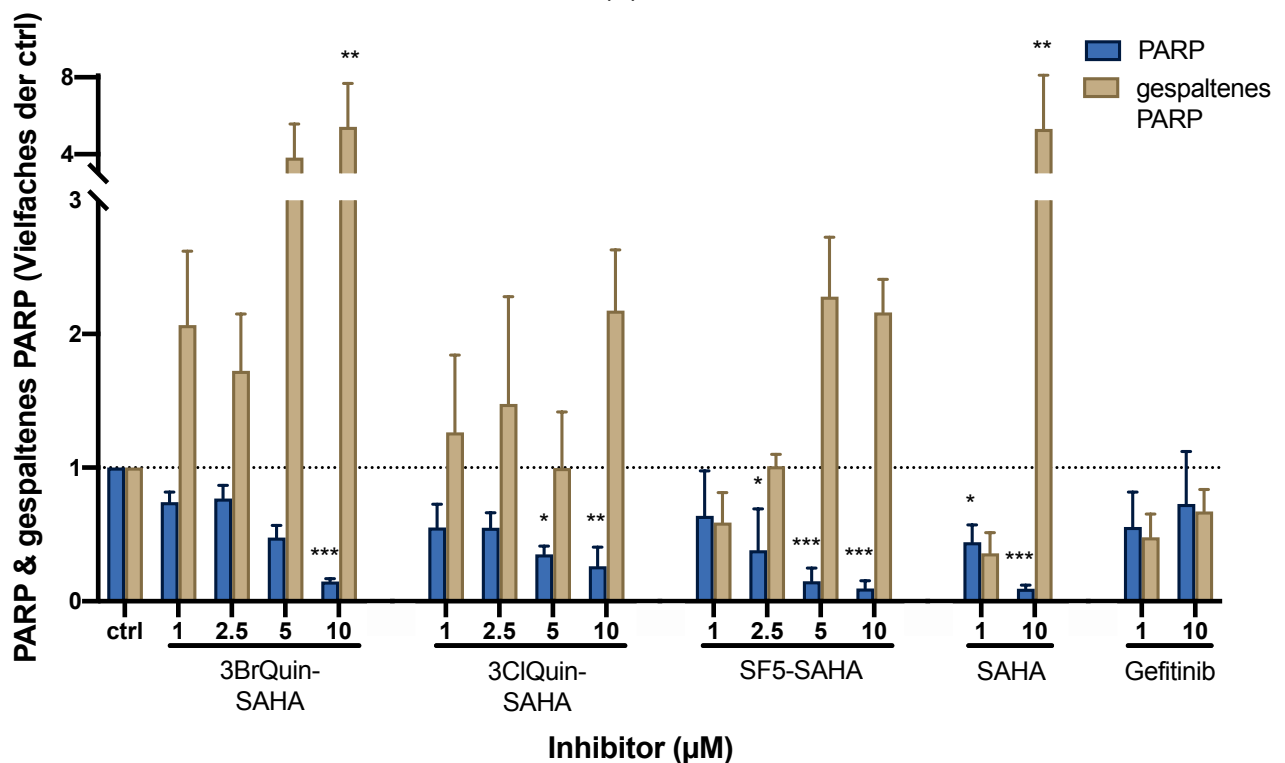
(b)

Abbildung 14: Caspase-3-Induktion durch die neuartigen Inhibitoren in den beiden Zellreihen (a) DU145 und (b) Hep-G2 gemessen nach unterschiedlichen Inkubationszeiten (6 h, 24h, 48 h) mit 3BrQuin-SAHA, 3CIQuin-SAHA, SF5-SAHA und den Kontrollsubstanzen SAHA und Gefitinib. Die Caspase-3-Aktivität wurde ins Verhältnis zu unbehandelten Kontrollzellen gesetzt und als Mittelwerte \pm SEM aus $n = 3$ unabhängigen Experimenten abgebildet. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$, **** $p \leq 0.0001$; two-way ANOVA Dunnett's post hoc test. Modifiziert aus [73, 78].

Als weiterer Marker für Chemotherapie-induzierte Apoptose wurden die Spaltung von Poly(ADP-Ribose)-Polymerasen (PARP) in sein kleineres ~ 85 kDa-Fragment, durch Caspase-3, in Western Blots von DU145 Zellen untersucht (Abbildung 15) [90, 91]. PARP-Spiegel verhalten sich indirekt proportional zu steigenden Konzentration von 3BrQuin-SAHA, 3CIQuin-SAHA, SF5-SAHA und SAHA, während sein Spaltprodukt ansteigt. Keine Änderungen wurden unter Gefitinib-Applikation beobachtet.



(a)



(b)

Abbildung 15: PARP-Spaltung durch die neuartigen Inhibitoren abgebildet durch einen (a) repräsentativen Western-Blot aus $n = 3$ Experimenten, der Veränderungen in der Expression von

PARP und dessen Spaltprodukt (c. PARP) in DU145-Zellen nach 24-stündiger Behandlung mit 3BrQuin-SAHA, 3ClQuin-SAHA, SF5-SAHA, SAHA und Gefitinib zeigt. β -Aktin wurde als Beladungskontrolle verwendet. (b) Die Grauintensitäten der Banden aus jeweils $n = 3$ Western Blots wurden quantifiziert, an die β -Aktin Beladungskontrolle angepasst und ins Verhältnis zur als 1 gesetzten Kontrolle gesetzt. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$; two-way ANOVA Dunnett's post hoc test. Modifiziert aus [73, 78].

3.6 HDAC-Inhibitor vermittelte Inhibition der Migration von PCa-Zellen

Zur Detektion behandlungsinduzierter Veränderungen im Migrationsverhalten der Zellen wurden Scratch-Assays durchgeführt (Abbildung 16). Ein verlangsamter Wiederverschluss einer im Zellrasen durch eine 100 μ l Pipettenspitze gesetzte Wunde (scratch), dient hierbei als read-out für das Migrationsverhalten der Zellen [92]. Um Migration und nicht Proliferation abzubilden, wurden Zeitpunkte gewählt, die unterhalb bzw. nicht höher als die durchschnittliche Verdopplungszeit der Zellkulturen liegen (3 – 24 h). Aus der qualitativen Auswertung der Aufnahmen geht hervor, dass 3ClQuin-SAHA und SF5-SAHA dosisabhängig über den Zeitraum von 24 h das Wanderungsverhalten von PCa (DU145) Zellen moderat beeinträchtigen.

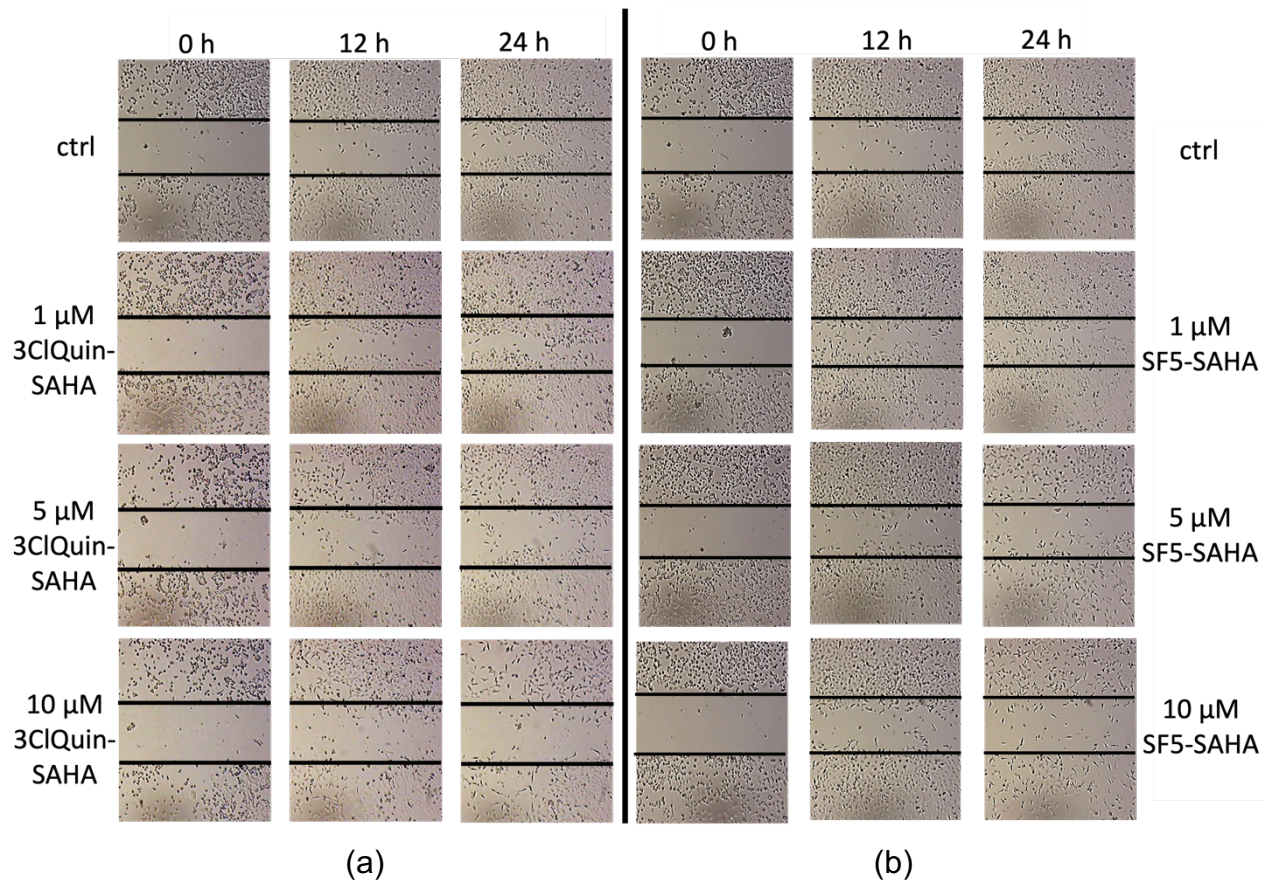


Abbildung 16: Inhibition der Migration von DU145 Zellen durch (a) 3CIQuin-SAHA und (b) SF5-SAHA. Die anfängliche Kratzfläche (0 h) dient als Referenzpunkt und wurde durch schwarze Linien hervorgehoben. Die Abbildungen repräsentieren Aufnahmen aus n = 3 unabhängigen Experimenten. Modifiziert aus [73, 78].

3.7 In vivo / in ovo Experimente zu den antiangiogenen Eigenschaften der dualen Inhibitoren

Um die neuartigen HDAC inhibierenden Substanzen erstmalig in einem in vivo Setting zu testen, wurde ein etabliertes in ovo Experiment zu den antiangiogenetischen Effekten der Inhibitoren durchgeführt (Abbildung 17) [93]. Die stark vaskularisierte CAM von befruchteten Hühnereiern wurde für drei Tage mit Inhibitoren behandelt und anschließend mit einer unbehandelten PBS-Kontrolle verglichen. Gesucht wurde nach einer Reduktion im Gefäßnetzwerk, sowie nach Dymorphologien einzelner Gefäße. 3BrQuin-SAHA und 3CIQuin-SAHA schienen dabei stärkere Effekte auf die Angiogenese als SAHA und Gefitinib zu haben, wobei 3CIQuin-SAHA die am stärksten ausgeprägten Effekte auf die Angiogenese zu haben scheint. Neben Beobachtungen zur Angiogenese ist zu erwähnen, dass die Inhibitoren während der Dreitagesbehandlung zu keinerlei erhöhter Mortalität der Hühnerföten führten.

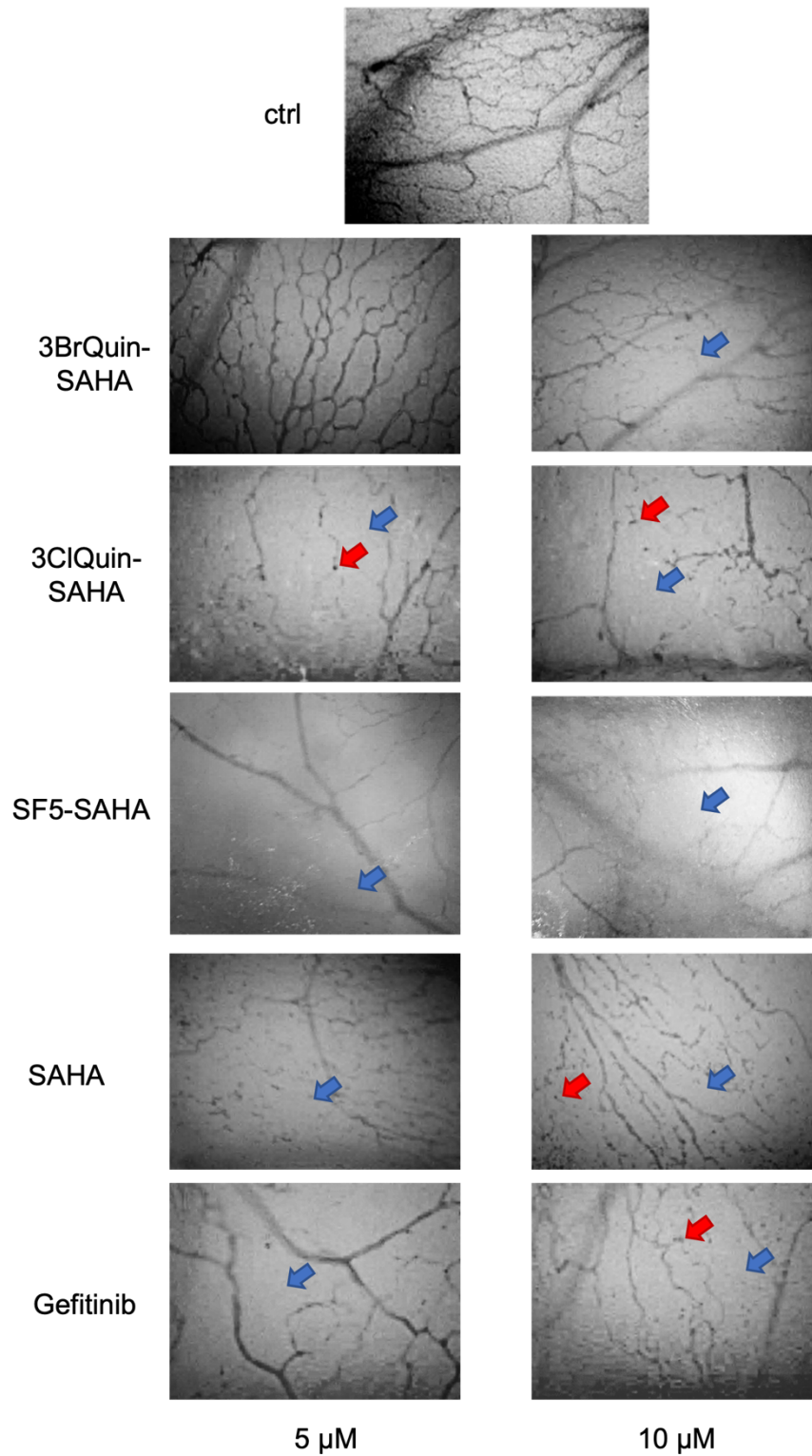


Abbildung 17: Antiangiogene Wirkungen von 3BrQuin-SAHA, 3ClQuin-SAHA und SF5-SAHA. Abgebildet werden Veränderungen im Mikrogefäßbett der CAM befruchteter Hühnereier nach 72-stündiger topischer Behandlung mit 3BrQuin-SAHA, 3ClQuin-SAHA und SF5-SAHA, sowie SAHA und Gefitinib als Vergleichssubstanzen. Zu beobachten ist eine Verringerung der Anzahl von Gefäßen (blauer Pfeil) mit Veränderungen der Morphologie (roter Pfeil). Die ausgewählten Bilder repräsentieren Ergebnisse aus Experimenten mit $N \geq 3$ Eiern. Modifiziert aus [73, 78].

4. Diskussion

Tumorerkrankungen sind die zweithäufigste Todesursache weltweit und es bedarf weiterer Verbesserungen der Therapien [2, 3]. Am Beispiel von PCa und HCC ist dies hinsichtlich mangelnder Alternativen zur Behandlung von Patient:innen im fortgeschrittenen Stadium der Erkrankung durch Resistenzentwicklung gegenüber der Erstlinientherapie beschrieben [94, 95]. In dieser Arbeit wurden, mit Bezug auf das vorangehend Gesagte, neuartige Substanzen auf ihre Wirksamkeit bei den beiden Tumorentitäten erstmals charakterisiert, um ihre grundsätzliche Eignung als neue, dringend benötigte Therapeutika präklinisch abschätzen und einordnen zu können.

Aus einer Substanzbibliothek wurden der erstmalig synthetisierte, pentafluorothio-substituierte HDAC-Inhibitor **SF5-SAHA** und die beiden neuen Tyrphostin AG1478- und Indazole-Derivate, gekoppelt an einen Hydroxamsäurerest, **3BrQuin-SAHA** und **3CIQuin-SAHA** ausgewählt und auf ihre antineoplastischen Eigenschaften untersucht. 3BrQuin-SAHA und 3CIQuin SAHA sind Dualinhibitoren, die aus einem HDAC inhibierenden Anteil (Hydroxamsäurerest) bestehen, der an einen Tyrosinkinaseinhibitor gegen den EGFR (Tyrphostin- und Indazole-Derivate) gekoppelt ist.

4.1 Zusammenfassung der Ergebnisse mit Einbettung in den aktuellen Stand der Forschung

Alle drei Inhibitoren haben starke antiproliferative Effekte im niedrigen mikromolarem Konzentrationsbereich auf DU145 (PCa) Zellen und Hep-G2 (HCC) Zellen gezeigt (siehe Tabelle 3 und Abbildung 8). Dabei scheinen die Inhibitoren keine allgemein zytotoxische Wirkung zu haben, sondern in signalwegspezifische Prozesse einzugreifen (siehe Abbildung 9). Eine erhöhte Zellproliferation und kompensatorische Hemmung der Apoptose sind maßgeblich an der Entwicklung von Krebs beteiligt [96]. Caspase-3 gehört zu den Apoptose induzierenden Effektorcaspasen und kann als positiver Marker für die Wirksamkeit von Chemotherapeutika in Tumoren betrachtet werden [97]. 3BrQuin-SAHA, 3CIQuin-SAHA und SF5-SAHA induzieren die Aktivierung von Caspase-3 dosis- und zeitabhängig in DU145 und Hep-G2 Zellen, mit einem gemessenen Maximum nach 24-stündiger Inkubation (siehe Abbildung 14). Konkordant dazu verhielt sich die PARP-Expression abnehmend in Western Blot Untersuchungen, durch dessen Caspase-3-induzierte Spaltung (siehe Abbildung 15) [90]. Eine zeitlich latente Erhöhung der Caspase-Aktivität

nach Applikation von Wasserstoffperoxid konnte bereits in Jurkat T-Lymphozyten beobachtet werden und lässt vermuten, dass die Bildung von ROS als Auslöser für Apoptose dienen kann [89, 98]. Alle untersuchten neuartigen Inhibitoren förderten die Bildung von ROS in DU145 und Hep-G2 Zellen bei Konzentration zwischen 1 - 10 μM stärker als die Referenzsubstanzen Gefitinib und SAHA bei äquimolaren Konzentrationen (siehe Abbildung 13). In HDAC resistenten Leukämie Zellen wurde gezeigt, dass SAHA-Resistenz durch vermehrte H_2O_2 Bildung, provoziert durch Glutathion (GSH) Depletion unter β -phenylethyl isothiocyanate Gabe, überwunden werden konnte [99]. Diese Erkenntnisse könnten auch für die neuartigen Inhibitoren eine Rolle spielen.

Weitere spezifische Wirkmechanismen wurden bezüglich Tyrosinkinase- und HDAC-Inhibition untersucht. Die neuartigen chimären Inhibitoren 3BrQuin-SAHA und 3ClQuin-SAHA hemmen die EGFR Kinaseaktivität im niedrigen mikromolaren Bereich und alle drei untersuchten Substanzen besitzen eine stark hemmende Wirkung auf die HDAC-Aktivität (siehe Abbildung 10). SF5-SAHA weist als reiner HDAC-Inhibitor das stärkste inhibitorische Potenzial, ähnlich das des klinisch relevanten Therapeutikums SAHA, auf. Erstaunlicherweise verminderten sowohl die Dualinhibitoren als auch SF5-SAHA und SAHA die Expression des EGFR in DU145 Zellen, was vermuten lässt, dass der HDAC-inhibitorische Hydroxamsäurerest der Substanzen regulatorisch auf die Expression des EGFR wirkt (siehe Abbildung 11). Bei gemeinsamer Betrachtung der Ergebnisse deuten diese also darauf hin, dass die chimären Inhibitoren einen dualen regulatorischen Charakter, sowohl über die Hemmung der Kinaseaktivität als auch über die Verminderung der Expression des Rezeptors, auf den EGF Signalweg haben. Bei genauerer Analyse der Literatur zeigte sich, dass Chou et al. selbiges Phänomen in verschiedenen Kolonkarzinomzelllinien sowie Hautkrebs- und Brustkrebszelllinien beobachteten [100].

Die Wirkung auf HDAC 2 und HDAC 6 wurden in separaten Assays genauer untersucht und deckte sich mit bisherigen Untersuchungen zur allgemeinen HDAC-Inhibition (siehe Abbildung 12). Beide HDACs werden durch 3BrQuin-SAHA, 3ClQuin-SAHA und SF5-SAHA (1 - 10 μM) stark gehemmt. Quint et al. stellten in ihrer Arbeit HDAC 2 als unabhängigen Prädiktor für das Überleben bei HCC-Patient:innen vor, da sie beobachteten, dass Patient:innen mit Zustand nach kurativer Leberchirurgie (M0 Status) mit hohem HDAC 2 immunoreactivity score (IRS, > 4 von 6) signifikant kürzer überlebten [59]. Kongruent dazu beschrieben Weichert et al., dass eine hohe HDAC 2 Expression (IRS \geq 7 von 12) eine unabhängige prognostische Bedeutung für das Gesamtüberleben von PCa Patienten mit Zustand nach radikaler Prostatektomie hat. Die Patienten mit geringer

HDAC 2 Expression (IRS ≤ 6 von 12) hatten eine signifikant höhere Wahrscheinlichkeit für ein 7-Jahre krankheitsfreie Überlebensrate (disease-free survival = DFS) und im Durchschnitt eine signifikant längere PSA-rezidivfreie Zeit [56]. 3CIQuin-SAHA und SF5-SAHA hemmten in vitro das Migrationsverhalten von DU145 Zellen, da sie verhinderten, dass Zellen in den Spalt, einer in einen konfluenten Zellrasen gesetzten „Wunde“, hinein migrierten (siehe Abbildung 16). In einer ersten systemischen Untersuchung wurden die neuartigen Inhibitoren direkt auf das sich entwickelnde Gefäßbett der CAM befruchteter Hühnereier appliziert und die Veränderung der Gefäßaussprossung beobachtet (siehe Abbildung 17). 3BrQuin-SAHA, 3CIQuin-SAHA und SF5-SAHA zeigten eine ausgeprägte antiangiogene Wirkungen auf das vaskuläre Netzwerk der CAM, ohne dabei das Überleben der sich entwickelnden Hühnerembryonen zu beeinträchtigen. In Kombination mit den Ergebnissen der LDH-Versuche sind dies experimentelle Hinweise zur Verträglichkeit von 3BrQuin-SAHA, 3CIQuin-SAHA und SF5-SAHA.

4.2 Relevanz von HDAC- und EGFR-Expressionsstatus in PCa und HCC

Die Überexpression von HDACs und EGFR sind sowohl im PCa als auch im HCC beschrieben worden [56-59, 70-72]. Freese et al. konnten in ihrer Untersuchung von HCC Gewebeproben aus Patient:innen mit Zustand nach partieller Hepatektomie, eine hohe Expression von HDAC der Klasse I (HDAC 1 und 2) und der Klasse II (HDAC 4,7 und 9) signifikant mit einer schlechteren Überlebenswahrscheinlichkeit korrelieren [57]. Konkordant dazu beobachteten Weichert et al., dass Prostatagewebeproben aus Patienten nach radikaler Prostatektomie eine erhöhte Expression von HDAC 1, 2 und 3 aufweisen, entdifferenzierter sind, höhere proliferative Anteile besitzen (gemessen am Ki-67 Index) und signifikant (nur HDAC 1 und 2) mit einem erhöhtem Gleason Score einhergehen [56]. Auch die Relevanz des EGFR wurde bereits von anderen Forschungsgruppen für das PCa und HCC untersucht. Ito et al. stellten fest, dass HCC-Proben aus Patienten nach chirurgischer Intervention, die vermehrt EGFR exprimierten, signifikant schlechter differenziert und proliferativ aktiver waren sowie öfter intrahepatische Metastasen aufwiesen. Für Patient:innen, die nach kurativer Chirurgie weiter beobachtet werden konnten, korrelierte der EGFR Status mit kürzeren DFS [70]. In Analogie dazu konstatierten Schlomm et al., dass erhöhte EGFR-Expressivität in Prostata Gewebe nach radikaler Prostatektomie mit erhöhtem Gleason-Score, einem fortgeschrittenem Tumorstadium, erhöhten

präoperativen PSA-Werten und kürzerem Überleben assoziiert ist [72]. Außerdem beobachteten Hernes et al., dass die Kastrationsresistenz von PCa signifikant mit vermehrter EGFR-Positivität einhergeht [71].

4.3 Vorteile einer Kombinationstherapie aus HDAC- und EGFR-Inhibitoren

Aus den synergistischen Effekten einer Kombination von HDAC und EGFR-Inhibition, wie sie beispielhaft am EGF-Signalweg beobachtet wurden, erhofft man sich bessere therapeutische Effekte sowie weniger Therapieresistenzen, da HDAC-Inhibitoren Resistenzmechanismen wie „Gen-silencing“ entgegenwirken [65, 66]. Zumal Resistenzentwicklung unter EGFR- Therapie ein bekanntes Problem in der Behandlung von Patient:innen mit Lungenkarzinom ist und sich das progressionsfreie Überleben (progression free survival = PFS) auf 9,6 - 13,1 Monate unter Gefitinib/Erlotinib/Afatinib Applikation beschränkt [101]. Nakagawa et al. untersuchten EGFR mutierte nicht-kleinzellige Lungenkarzinom (NSCLC) Zelllinien, die aufgrund eines BIM (B cell lymphoma-2-like 11 (BCL2L11)) Deletion Polymorphismus resistent gegenüber der Therapie mit Gefitinib sind. Eine Kombinationstherapie aus Gefitinib und SAHA konnte die Expression von BIM wiederherstellen und die Gefitinib-Resistenz überwinden [102]. Weiterführend untersuchten Takeuchi et al. in der VICTORY-J Phase I Studie die Kombination aus Gefitinib und Vorinostat an Patient:innen mit EGFR mutierten NSCLC mit BIM Deletion Polymorphismus, die eine Tumorprogression unter Therapie eines EGFR-Inhibitoren (Gefitinib, Erlotinib oder Afatinib) und Chemotherapie aufwiesen. An 12 Patient:innen wurde die Kombination getestet und eine Applikationsdosis von 250 mg Gefitinib täglich und 400 mg/Tag Vorinostat zweimal pro Woche empfohlen. Das PFS betrug 5,2 Monate und das Gesamtüberleben (overall survival = OS) 22,4 Monate. Die Krankheits-Kontroll Rate (disease control rate = DCR) über mindestens sechs Monate betrug 83,3 %. In weiteren Untersuchungen entdeckte die Forschungsgruppe, dass die Kombinationstherapie aus Gefitinib und Vorinostat einen Anstieg des proapoptotischen BIM Exon 4 und eine Erniedrigung des BIM Exon 3/Exon 4 Verhältnisses in Blutanalysen der Proband:innen verursachte. Der Acetylierungsstatus von Histon H3, einem Marker für HDAC-Inhibition, war in 10 von 11 Proben erhöht [103]. Weitere Arbeitsgruppen konnten in Zellversuchen anderer Tumorentitäten eine Resistenzüberwindung gegen EGFR-Inhibitoren unter Hinzunahme von HDAC-Inhibitoren beobachten [104-106].

In einer weiteren Studie untersuchten Freese et al. wie sich eine Kombinationstherapie aus Sorafenib und SAHA auf HCC-Zelllinien auswirkt. Sie beobachteten einerseits, dass Sorafenib und SAHA synergistisch Zelltod und Apoptose induzieren und andererseits, dass diese Kombination Sorafenib Resistenz in Hep3B Sorafenib-resistenten Zellen überkommt [57].

In der klinischen Phase I/II SHELTER Studie von Bitzer et al. profitierten Patient:innen mit Sorafenibresistenz bei HCC von einer Kombinationstherapie aus Sorafenib (400 mg) und dem HDAC-Inhibitor Resminostat (600 mg) gegenüber einer Monotherapie mit Resminostat (600 mg), gezeigt an einem längeren PFS (6,5 vs. 1,6 Monate), durchschnittlich längerem OS (8,0 vs. 4,1 Monate) und einer besseren DCR (87,5 % vs. 43,8 %) [107].

4.4 Klinische Studien zum Einsatz von HDAC-Inhibitoren beim HCC

HDAC-Inhibitoren sind bereits seit längerem etablierte Medikamente in der Therapie von Tumorerkrankungen des hämatoonkologischen Formenkreises und derzeitig Bestandteil zahlreicher Phase I, II und III Studien für Tumoren unterschiedlicher Entitäten, inklusive solider Tumore, als Einzelsubstanzen oder in Kombinationstherapien [41-46, 108-110].

In der klinischen Phase I/II Studie von Tak et al. wurde die Behandlung des HDAC-Inhibitor Resminostat + Sorafenib mit der einer Monotherapie mit Sorafenib als Erstlinientherapie bei Patient:innen mit fortgeschrittenen HCC verglichen. Es konnte kein signifikanter Unterschied für die Zeit bis zur (Tumor) Progression (time to progression = TTP) und das OS zwischen den beiden Therapieansätzen gefunden werden. Subgruppenanalysen lassen aber vermuten, dass eine Kombinationstherapie positive additive Effekte bei der Therapie bestimmter Patient:innen Gruppen wie z.B. Patient:innen aus Korea, Patient:innen mit Hepatitis B und Patient:innen mit erhöhten Ausgangswerten der Blutplättchen ($\geq 150 \times 10^3/\text{mm}^3$) haben könnte [111]. Die bereits erwähnte SHELTER Studie von Bitzer et al. zeigte jedoch, dass Patient:innen mit Sorafenibresistenz von einer Zweitlinientherapie mit Resminostat (600 mg) plus Sorafenib (400 mg) profitieren könnten [107]. In einer früheren Studie von Yeo et al. wurde bereits die Wirksamkeit des HDAC-Inhibitors Belinostat bei 42 Patient:innen mit nicht resektablem HCC getestet. Bei einer applizierten Dosis von 1400 mg/m^2 Belinostat pro Tag betrug das PFS 2,64 Monate (95 % CI, 1,55 bis 3,17 Monate) und das OS 6,6 Monate. In dieser Studie gibt es keine Vergleichskohorte, die Autor:innen postulieren jedoch, dass das PFS unter Standardtherapie nur 1,4

Monate beträgt. Aufgrund der fehlenden Vergleichskohorte lassen die Ergebnisse keine endgültigen Aussagen über die Effektivität von Berlinostat bei Patient:innen mit nicht resektablem HCC zu. Interessant ist aber, dass bei der Untersuchung von 38 vor Therapie gewonnener Gewebeproben eine hohe Expression von HR23B signifikant mit einer besseren Krankheitskontrolle gegenüber Patient:innen mit geringer Expression korreliert werden konnte [112]. HR23B wurde bereits zuvor als möglicher prädiktiver Biomarker für das Ansprechen auf die Therapie mit HDAC-Inhibitoren vorgestellt [113].

4.5 Klinische Studien zum Einsatz von HDAC-Inhibitoren beim PCa

Für die Therapie des PCa mit HDAC-Inhibitoren als Monotherapie wurden bereits mehrere klinische Phase II Studien an Patient:innen mit CRPC durchgeführt. Die untersuchten Medikamente blieben trotz vielversprechender präklinischer Daten hinter den Erwartungen bezüglich klinischer antitumoraler Effekte zurück [114-117]. In der Phase II Studie von Bradley et al. wurde die Wirksamkeit von SAHA (400 mg/d p.o.) an Patienten mit metastasiertem Prostatakarzinom und Krankheitsprozess unter Androgendeprivations-therapie sowie vorangegangener Chemotherapie geprüft. Es wurde keine klinisch bedeutsame Wirksamkeit (7 % stabile Erkrankung (stable disease = SD)) nach Response Evaluation Criteria in Solid Tumors criteria (RECIST Kriterien) beobachtet und bei 41 % der Patienten wurde die Therapie aufgrund von Nebenwirkungen vorzeitig abgebrochen, was eine Aussage bezüglich der Wirksamkeit von SAHA in dieser Studie erschwert [114, 118]. Molife et al. applizierten den HDAC-Inhibitor Romidepsin (13 mg/m² i.v.) Chemotherapie naiven CRPC-Patienten, wodurch bei 5,7 % ein radiologisches partielles Ansprechen (partial response = PR) nach RECIST Kriterien mit ≥ 50 % PSA-Verminderung und bei 31 % SD, bei einer TTP von 49,5 Tagen, erreicht werden konnte [115]. Ein weiterer HDAC-Inhibitor Panobinostat (20 mg/m² i.v.) wurde von Rathkopf et al. an Patienten mit metastasierten CRPC und Krankheitsprogression nach zytotoxischer Therapie untersucht. Der primäre Endpunkt an PFS nach 24 Wochen wurde nur von 11,4 % der Patienten erreicht [116]. Als weitere klinische Phase II Studie mit einem HDAC-Inhibitor als Monotherapie bei Patienten mit progressivem CRPC wurde die Wirksamkeit von Pracinostat (60 mg/Tag p.o.) untersucht. Nur bei 6 % der Patienten wurde eine PSA-Reduktion von ≥ 50 % gemessen [117].

Aufgrund der epigenetisch regulatorischen Wirkweise von HDAC-Inhibitoren ist deren Einsatz in Kombinationstherapien trotz eingeschränkter Wirkung als Monotherapie von

besonderem Interesse. Ferrari et al. untersuchten in ihrer Phase I/II Studie, wie sich eine Kombinationstherapie aus dem Antiandrogen Bicalutamide (50 mg/Tag p.o.) in Kombination mit dem HDAC-Inhibitor Panobinostat auf Patienten mit CRPC auswirkt, die sich unter Applikation des ersten antiandrogenen Zweitlinientherapeutikums (2ndLAARx) weiterhin tumorprogredient verhielten, nachdem sie in präklinischen Studien synergistische Effekte dieser beiden Therapeutika beobachten konnten. Außerdem konnte durch die kombinatorische Gabe die Androgenresistenz gegenüber Bicalutamide in der androgenunabhängigen Zelllinie LNCaP überwunden werden und in den kastrations- und antiandrogenresistenten Prostatakrebs-Zelllinie 22Rv1 und Xenograft CWR22PC reproduziert werden [119, 120]. Den Patienten der zweiarmigen Studie wurde entweder eine dreimal wöchentliche Dosis von 40 mg (A Arm) oder 20 mg (B Arm) Panobinostat über 2 - 3 Wochenzyklen zusammen mit 50 mg/Tag Bicalutamide verabreicht. Da es keinen Kontrollarm gibt, beziehen sich die Autor:innen dieser Studie auf ältere Studien zu Bicalutamide.

(1) Patienten mit androgenunabhängigen Tumoren, dessen Tumorerkrankung sich progredient unter einer Therapie mit Flutamid (2ndLAARx) als Teil einer kombinierten Androgenblockade mit einem GnRH-Analogon (1ndLAARx), erhielten Bicalutamide (200 mg/Tag). Die durchschnittliche Zeit bis zur PSA-Progression, definiert als 50 % Anstieg zu Baseline, betrug 3 - 4 Monate [121].

(2) Patienten mit nichtmetastasierten oder metastasierten CRCP einer 1:1 randomisierten, doppelt verblindeten Phase II Studie zur Wirksamkeit von Enzalutamide versus Bicalutamide mit Tumorprogress unter Androgendeprivationstherapie (ADT) erhielten 50 mg Bicalutamide zusätzlich zur ADT. Die durchschnittliche Zeit in der Kohorte mit metastasiertem CRPC bis zur PSA-Progression (> 25 % Anstieg zur Baseline nach Prostate Cancer Clinical Trials Working Group 2 (PCGW2)-Richtlinien [122]) betrug 5,7 Monate und bis zum radiologischem PFS (rPFS) 8,3 Monate. Das Vorliegen von Metastasen wurde aufgrund besserer Vergleichbarkeit mit der Studienpopulation (94,5 % mCRPC) gewählt. Die Differenz zwischen gemessenem PSA-Anstieg und radiologischem Progress betrug 2,6 Monate [123].

Eine Therapie mit 40 mg Panobinostat plus 50 mg/Tag Bicalutamide (A Arm) erzielte durchschnittlich ähnliche lange Werte bis zur PSA-Progression, definiert als 50 % Anstieg zu Baseline, wie in der Vergleichsstudie (1) (15 vs. 16 Wochen). Jedoch betrug die Bicalutamide Dosis nur 50 mg statt 200 mg und Patienten wurden bereits erfolglos mit mehr als nur 1 Antiandrogen behandelt. In Bezug auf die Studie (2) demonstrierten Ferrari et

al., dass durch ihre Hochdosiskombinationstherapie eine radiologische Progression erst nach 33,9 Monaten im Gegensatz zu 8,3 Monaten unter Bicalutamide Monotherapie gesehen wurde. Auch das Intervall zwischen PSA-Anstieg ($> 25\%$ Anstieg zur Baseline nach PCGW2 [122]) und rPFS viel deutlich länger aus ($33,9 - 9,4 = 24,5$ Wochen vs. $8,3 - 5,7 = 2,6$ Wochen). In der Studie wurde als primärer Endpunkt die Bestimmung des Anteils der Patienten, die nach 9 Monaten Behandlung radiologisch progressionsfrei (rPF) waren, definiert. Die errechneten Patientenanteile von $47,5\%$ für Arm A und $38,7\%$ für Arm B überstiegen das vor Studienbeginn als ein klinisch relevantes einzustufende Ergebnis von $> 35\%$ rPF für beide Studienarme. Die Autor:innen zeigten, dass signifikante Ergebnisse hauptsächlich in dem A arm mit Hochdosis (40 mg) Panobinostat erzielt wurden, gleichzeitig jedoch vermehrter Dosisreduktionen aufgrund von Nebenwirkungen vorgenommen werden mussten, was eine geringe therapeutische Breite der Kombination aus Panobinostat und Bicalutamide vermuten lässt. Diese erfolgreiche klinische Studie sollte mit dem wirksameren zweite Generation Antiandrogen wie z.B. Enzalutamide wiederholt werden [120].

4.6 CUDC-101 ein potenter multitarget EGFR/HER2/HDAC-Inhibitor

Der Therapieansatz aus Dualinhibitoren mit EGFR und HDAC inhibitorischen Anteilen, wie er durch 3BrQuin-SAHA und 3ClQuin-SAHA in dieser Arbeit repräsentiert wird, ist Bestandteil der Untersuchungen anderer Forschungsgruppen. Der von Cai et al. vorgestellte duale Kinase/HDAC-Inhibitor 7-(4-(3-Ethynylphenylamino)-7-methoxyquinazolin-6-yloxy)-N-hydroxyheptanamide (**CUDC-101**) ist ein potenter multitarget EGFR/HER2/HDAC-Inhibitor, der bereits in verschiedenen klinischen Studien an Patienten mit soliden Tumoren unterschiedlicher Entitäten getestet wird [76]. In den ersten veröffentlichten Studien der Arbeitsgruppe zur Synthese und Charakterisierung von CUDC-101 wurde untersucht, wie dieser das Zellwachstum in vitro von diversen Zelllinien, unter anderem auch Hep-G2 (HCC) und PCa beeinflusst. Interessant ist, dass CUDC-101 das Zellwachstum in Proliferationsversuchen zur Bestimmung des IC_{50} stärker hemmt als eine Kombination aus SAHA (HDAC-Inhibitor) und einem EGFR-Inhibitor wie Erlotinib oder Lapatinib [76, 77]. Diese Studien sprechen für die Überlegenheit einer Therapie mit dualen Inhibitoren über der einer Kombinationstherapie, bei der zwei Einzelsubstanzen verabreicht werden. Die Wechselwirkungen zwischen den Einzelsubstanzen gilt es weiter abzuklären [74]. In Zellversuchen von Wang et al. konnte zusätzlich gezeigt werden, dass

CUDC-101 EGFR-Resistenzen in NSCLC überkommen kann und eine längere Gabe von CUDC-101 in niedrigen Dosen, anders als bei langer Erlotinib und Gefitinib Gabe, keine Resistenzentwicklung fördert [124]. In der ersten Phase I Studie von Shimizu et al. zur Ermittlung der maximal tolerierbaren Dosis (MTD) von CUDC-101 wurde der chimäre Inhibitor 25 Patient:innen mit soliden Tumoren, die sich refraktär gegenüber der Standardtherapie verhielten oder keine solche existierte, verabreicht. Nach dem 3 + 3 Dosisescalationsschema wurde CUDC-101 als 1-stündige Infusion an fünf aufeinanderfolgenden Tagen alle 14 Tage in fünf Kohorten verabreicht (Abbildung 18). Als MTD wurde 275 mg/m² definiert, da es in der 300 mg/m² bei 3/4 Patient:innen zum Auftreten dosislimitierender Toxizität (DLT) kam (1 mal Grad 3 Perikarditis, 2 mal Grad 2 Kreatininanstieg). In der 275 mg/m² Kohorte entwickelte 1/6 Proband einen Grad 2 Kreatininanstieg. Weitere unerwünschte Nebenwirkungen, die nicht der Kategorie dosislimitierender Toxizität zugeordnet wurden, waren vorübergehende reversible Übelkeit (24 %), Müdigkeit (24 %), trockene Haut (16 %), und Erhöhung der Serum-Aspartat-Aminotransferase (AST) (12 %).

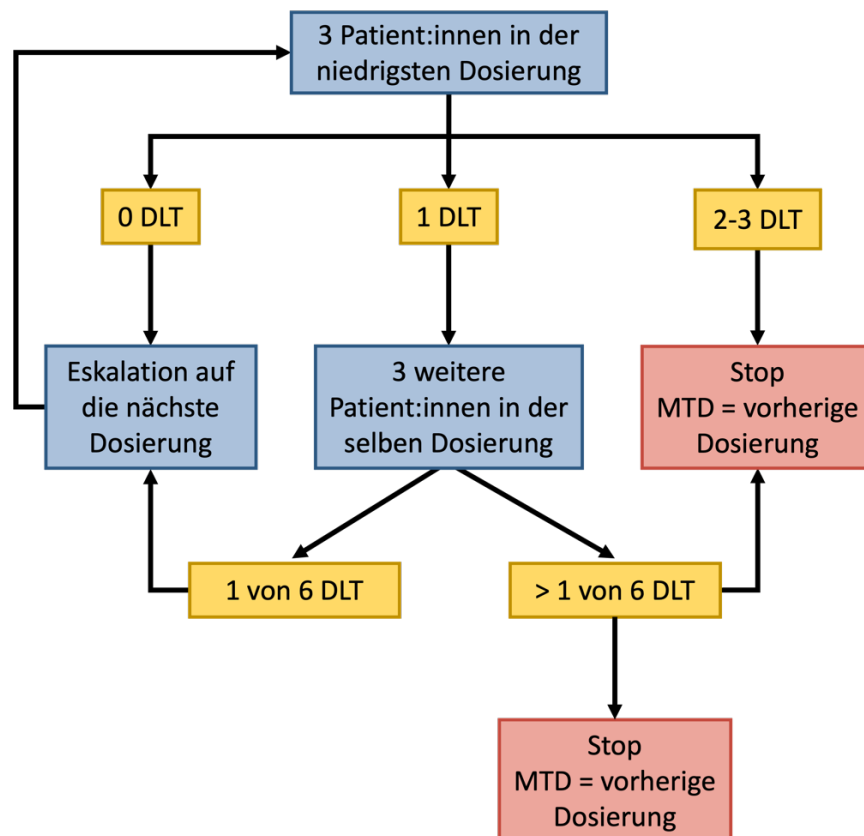


Abbildung 18: 3 + 3 Dosisescalationsschema zur Ermittlung der maximal tolerierbaren Dosis. MTD = maximal tolerierbare Dosis, DLT = dosislimitierende Toxizität. Modifiziert aus [125].

Die systemische Verfügbarkeit und Target spezifische Wirksamkeit des Pharmakons wurde von der Arbeitsgruppe durch Induktion von acetyliertem Histon H3 aus Hautproben von Patient:innen belegt. Vielversprechende Antitumoreffekte konnten bei unterschiedlichen Proband:innen beobachtet werden. Nach RECIST Kriterien erhielt ein Patient mit metastasiertem Magenkarzinom eine PR mit Tumorreduktion der Zielläsionen um 56 % und sechs weitere eine SD unter CUDC-101-Therapie [126]. Eine weitere Studie prüfte die Anwendung von CUDC-101 in Kombination mit Cisplatin-Radiotherapie zur klinischen Anwendung und Therapie von Plattenepithelkarzinomen von Kopf und Hals (head and neck squamous cell carcinoma = HNSCC), eine Tumorentität bei der berichtet wurde, dass in bis zu 38 - 47 % der Tumoren eine Überexpression an EGFR vorliegt, was mit schlechtem Therapieansprechen auf die konventionelle Strahlentherapie und vermindertem Gesamtüberleben einhergeht [127-129]. Andere Studien deuten darauf hin, dass selbiges für die Expression von HDAC I und II gilt [130, 131]. In der Studie von Galloway et al. wurden 12 Patient:innen mit lokal begrenzten, fortgeschrittenen HNSCC ohne systemische Vorbehandlung eingeschlossen. Beruhend auf der Studie von Shimizu et al. wurden die Patient:innen nach dem 3 + 3 Dosisescalationsschema mit CUDC-101 Dosen von 225 mg/m² oder 275 mg/m² im Verlauf einer Woche dreimal mit CUDC-101 behandelt, eine Woche vor Beginn der Radiotherapie und anschließend als kombinierten Behandlung in den Wochen 1 bis 7. Cisplatin (100 mg/m²) wurde alle drei Wochen verabreicht. Die MTD wurde auch in der Kombinationstherapie mit 275 mg/m² bestätigt, da nur einmalig DLT in Form einer Grad 3 Nierenläsion beobachtet wurde. Jedoch sollte die Empfehlung laut Autoren überdacht werden, da 4 der 5 Studienabrechenden aufgrund von nicht DLT unerwünschter Nebenwirkungen der 275 mg/m² Kohorte zugehörig waren. Nebenwirkungen, die zum Therapieabbruch von CUDC-101 führten waren: Grad 2 Gelenkschmerzen und Myalgie (n = 1), Grad 1 Kreatininanstieg (n = 1), Grad 5 Herzinsuffizienz (n = 1) und Grad 3 Extravasation der Infusion (n = 1). Weitere beschriebene schwere Nebenwirkungen aufgrund der CUDC-101 Behandlung waren Stomatitis (n = 1) und akutes Nierenversagen mit Nierenschädigung (n = 1). Weitere Nebenwirkungen, die nicht mit der CUDC-101 Applikation in Verbindung gebracht wurden, waren Müdigkeit (67 %), Stomatitis (67 %), Übelkeit (50 %) und Gewichtsverlust (50 %), sowie Gedeihstörung (n = 1), Flüssigkeitsüberladung (n = 1), Hyperglykämisches nichtketotisches hyperosmolares Syndrom (n = 1), Hypoxie (n = 1) und Pneumonie (n = 1), die als schwere Nebenwirkungen gewertet wurden. Zur Kontrolle der systemischen Verfügbarkeit wurden nach Behandlung Hautbiopsien entnommen, in denen EGFR-Inhibition und verstärkte Histon-

H3-Acetylierung beobachtet wurden. Erste Daten dieser Phase I Studienkohorte zeigen ein vielversprechendes Therapieansprechen. Acht Patient:innen konnten nach RECIST Kriterien beurteilt werden: Komplette Remission (complete response = CR) 62,5 %, PR 12,5 % und 25 % SD. Nach „Bewertung des klinischen Gesamtansprechens“ verbesserten sich 63 % und 37 % stabilisierten sich unter der Therapie. 90 % der Proband:innen waren nach einer durchschnittlichen Nachbeobachtungszeit von 1,47 Jahren progressionsfrei [132].

Zur Tumorentität des PCa gibt es eine in vitro + in vivo Studie von Sun et al., die die Wirksamkeit von CUDC-101 in CRPC untersucht. Ursächlich für einen erneuten PSA-Anstieg unter antiandrogener Therapie, die sogenannte Kastrationsresistenz, sind Mutation des Androgen Rezeptors (fAR = Full Length Androgen Rezeptor) zu Androgen Rezeptor Variante, wie z.B. AR-V7 [133]. Durch Messung von PSA mRNA, als Marker der AR-Aktivität, konnte gezeigt werden, dass CUDC-101 signifikant die Aktivität und Transkription von fAR und AR-V7 senkt. Weiterführend zeigte die Arbeitsgruppe, dass CUDC-101 signifikant, gegenüber unbehandelten Kontrollen, das Tumorwachstum in 22Rv1 Xenografts, einer CRCP-Zelllinie, hemmt [134, 135].

5. Schlussfolgerungen

In dieser Arbeit wurden die neu synthetisierten HDAC basierten Inhibitoren 3BrQuin-SAHA, 3ClQuin-SAHA und SF5-SAHA vorgestellt und in ihrer Wirksamkeit und Wirkweise an Prostata- und HCC-Karzinomzellen untersucht [73, 78]. HDAC-Inhibitoren, die durch eine Vielzahl an Mechanismen, antineoplastisch auf Tumoren wirken und zur Therapie von hämatologischen Karzinomen bereits zugelassen sind, bieten einen vielversprechenden Therapieansatz für Tumoren anderer Ätiologie und eignen sich gut als Kombinationspartner zur Entwicklung von chimären Inhibitoren, wie im Fall von 3BrQuin-SAHA und 3ClQuin-SAHA.

Die untersuchten Inhibitoren hemmen das *in vitro* Wachstum von DU145 und Hep-G2 Zellen im niedrigen mikromolaren Bereich. Hierbei wird in Signalwege der Histon deacetylierung und des EGFR (für 3BrQuin-SAHA und 3ClQuin-SAHA) eingegriffen. Weiterhin wurde gemessen, dass die neuartigen Inhibitoren Apoptose mittels Caspase-3-Induktion, sowie die Bildung von ROS, fördern, ohne dabei unspezifisch zytotoxisch auf die Zellen zu wirken. *In vivo/in ovo* haben die Substanzen antiangiogene Effekte [73, 78].

Diese vielversprechenden Ergebnisse der präklinischen Versuche legitimieren weitere Forschung, beispielsweise an zusätzlichen Tiermodellen, um die Wirksamkeit und Relevanz der neuen Substanzen besser einordnen zu können.

Bei gründlicher Betrachtung der Literatur, finden sich zahlreiche Studien, die die Wirksamkeit von HDAC-Inhibitoren an Zellversuchen und in klinischen Studien untersuchen. Aus Phase I und II Studien zu den Tumorentitäten des Prostata- und hepatozellulärem Karzinom zeigte sich, dass Patient:innen bei denen die Erstlinientherapie versagte, von einer Kombinationstherapie mit HDAC-Inhibitoren profitieren könnten [107, 120]. Der duale Kinase/HDAC-Inhibitor CUDC-101, der eine Ähnlichkeit zu den beiden Substanzen 3BrQuin-SAHA und 3ClQuin-SAHA dieser Arbeit aufweist, wurde in zwei Phase I Studien erfolgreich zur sicheren Anwendbarkeit bei solide Tumoren und HNSCC-Tumoren getestet [126, 132]. Die vielversprechenden Ergebnisse der Studien unterstützen weitere Untersuchungen zur klinischen Anwendbarkeit des Inhibitors.

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Eidesstattliche Versicherung

„Ich, Nils Göhringer, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: Untersuchungen zur antineoplastischen Wirkung neuartiger HDAC-Inhibitoren bei Prostata- und hepatozellulären Karzinomen; Studies on the antineoplastic effect of novel HDAC inhibitors in prostate and hepatocellular carcinomas selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet. Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.org) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum

Unterschrift

Anteilserklärung an den erfolgten Publikationen

Nils Göhringer hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Nils Goehringer, Bernhard Biersack, Yayi Peng, Rainer Schobert, Marco Herling, Andi Ma, Bianca Nitzsche und Michael Höpfner, Anticancer Activity and Mechanisms of Action of New Chimeric EGFR/HDAC-Inhibitors, International Journal of Molecular Sciences, 2021

Beitrag im Einzelnen: Planung; Umgang mit Zellkulturen; Methodik; Datenerhebung (Proliferationsversuche, LDH-Assay, HDAC-Assays, Caspase-3-Assay, ROS-Assay, Western Blots), Statistische Auswertung; Erstellen der Abbildungen; Visualisierung; Schreiben des Textes; Überarbeitung im Review-Prozess. Alle Abbildungen wurden von mir erstellt, außer Schema 1, Abbildung 4d und die Ergebnisse zu den T-Zell Lymphom Zelllinien aus der Abbildung 1d sowie der Tabelle 1.

Publikation 2: Nils Goehringer, Yayi Peng, Bianca Nitzsche, Hannah Biermann, Rohan Pradhan, Rainer Schobert, Marco Herling, Michael Höpfner und Bernhard Biersack, Improved Anticancer Activities of a New Pentafluorothio- Substituted Vorinostat-Type Histone Deacetylase Inhibitor, Pharmaceuticals, 2021

Beitrag im Einzelnen: Planung; Umgang mit Zellkulturen; Methodik; Datenerhebung (Proliferationsversuche, LDH-Assay, EGFR/HDAC-Assays, Caspase-3-Assay, ROS-Assay, Western Blots, Angiogenesversuche, Migrationsversuche), Statistische Auswertung; Erstellen der Abbildungen; Visualisierung; Schreiben des Textes.

Alle Abbildungen wurden von mir erstellt, außer Schema 1, Abbildung 1, 6, 9, 12, sowie die Western Blots mit Hep-G2 Zellen (aus Abbildung 3d, 5b und 8) und die Ergebnisse zu den T-Zell Lymphom Zelllinien aus der Tabelle 1.

Publikation 3: Andi Ma, Bernhard Biersack, Nils Goehringer, Bianca Nitzsche und Michael Höpfner, Novel Thienyl-Based Tyrosine Kinase Inhibitors for the Treatment of Hepatocellular Carcinoma, Journal of Personalized Medicine, 2022

Beitrag im Einzelnen: Umgang mit Zellkulturen; Hilfe bei Experimentenplanung, Datenerhebung und Visualisierung.

Unterschrift, Datum und Stempel des/der erstbetreuenden Hochschullehrers/in

Unterschrift des Doktoranden/der Doktorandin

Auszug aus der Journal Summary List

Publikation 1:

Journal Data Filtered By: **Selected JCR Year: 2020** Selected Editions: SCIE,SSCI
 Selected Categories: **"BIOCHEMISTRY and MOLECULAR BIOLOGY"** Selected
 Category Scheme: WoS
Gesamtanzahl: 297 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE MEDICINE	114,401	53.440	0.184050
2	CELL	320,407	41.582	0.526960
3	Molecular Cancer	24,931	27.401	0.030030
4	Annual Review of Biochemistry	24,394	23.643	0.021450
5	Signal Transduction and Targeted Therapy	3,848	18.187	0.005730
6	MOLECULAR CELL	86,299	17.970	0.161840
7	TRENDS IN MICROBIOLOGY	17,553	17.079	0.022820
8	NUCLEIC ACIDS RESEARCH	248,139	16.971	0.387070
9	MOLECULAR BIOLOGY AND EVOLUTION	61,557	16.240	0.082270
10	PROGRESS IN LIPID RESEARCH	7,328	16.195	0.004530
11	MOLECULAR PSYCHIATRY	28,622	15.992	0.046220
12	CELL DEATH AND DIFFERENTIATION	27,701	15.828	0.028730
13	NATURE STRUCTURAL & MOLECULAR BIOLOGY	32,038	15.369	0.051210
14	Nature Chemical Biology	27,428	15.040	0.047880
15	MOLECULAR ASPECTS OF MEDICINE	8,136	14.235	0.006640
16	TRENDS IN BIOCHEMICAL SCIENCES	22,003	13.807	0.025760
17	NATURAL PRODUCT REPORTS	13,293	13.423	0.011160
18	Molecular Plant	15,778	13.164	0.026860
19	Advances in Carbohydrate Chemistry and Biochemistry	752	12.200	0.000200
20	TRENDS IN MOLECULAR MEDICINE	13,213	11.951	0.014720

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
21	Redox Biology	15,982	11.799	0.024930
22	EMBO JOURNAL	76,189	11.598	0.055000
23	MATRIX BIOLOGY	8,972	11.583	0.011010
24	Molecular Systems Biology	10,149	11.429	0.016300
25	PLANT CELL	64,794	11.277	0.036260
26	CURRENT BIOLOGY	78,289	10.834	0.116100
27	BIOCHIMICA ET BIOPHYSICA ACTA-REVIEWS ON CANCER	7,025	10.680	0.007000
28	Cell Systems	5,813	10.304	0.035330
29	ONCOGENE	77,576	9.867	0.059180
30	CELLULAR AND MOLECULAR LIFE SCIENCES	34,003	9.261	0.033790
31	GENOME RESEARCH	47,141	9.043	0.064690
32	CURRENT OPINION IN CHEMICAL BIOLOGY	12,240	8.822	0.014190
33	EMBO REPORTS	19,502	8.807	0.027490
34	EXPERIMENTAL AND MOLECULAR MEDICINE	8,780	8.718	0.013260
35	ANTIOXIDANTS & REDOX SIGNALING	26,971	8.401	0.016700
36	CRITICAL REVIEWS IN BIOCHEMISTRY AND MOLECULAR BIOLOGY	4,576	8.250	0.005370
37	Science Signaling	15,954	8.192	0.023910
38	Cell Chemical Biology	5,236	8.116	0.018050
39	PLOS BIOLOGY	39,598	8.029	0.059920
40	Essays in Biochemistry	3,629	8.000	0.006450
41	BIOINORGANIC CHEMISTRY AND APPLICATIONS	1,406	7.778	0.000890

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
42	Acta Crystallographica Section D-Structural Biology	23,670	7.652	0.020190
43	CYTOKINE & GROWTH FACTOR REVIEWS	7,650	7.638	0.005850
44	FREE RADICAL BIOLOGY AND MEDICINE	52,714	7.376	0.034180
45	Computational and Structural Biotechnology Journal	3,620	7.271	0.006770
46	AMYLOID-JOURNAL OF PROTEIN FOLDING DISORDERS	2,202	7.141	0.003280
47	Cell and Bioscience	3,184	7.133	0.004320
48	Genes & Diseases	1,850	7.103	0.003170
49	Molecular Ecology Resources	13,390	7.090	0.016690
50	Journal of Integrative Plant Biology	6,749	7.061	0.006430
51	BIOMACROMOLECULES	45,724	6.988	0.026020
52	INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES	79,246	6.953	0.073720
53	AMERICAN JOURNAL OF RESPIRATORY CELL AND MOLECULAR BIOLOGY	15,280	6.914	0.015050
54	International Review of Cell and Molecular Biology	3,057	6.813	0.004320
55	CURRENT OPINION IN STRUCTURAL BIOLOGY	12,448	6.809	0.018970
56	PROTEIN SCIENCE	16,581	6.725	0.021220
57	International Journal of Biological Sciences	10,778	6.580	0.010540
58	Open Biology	4,059	6.411	0.010280
59	MOLECULAR MEDICINE	6,239	6.354	0.004460
60	Antioxidants	9,076	6.312	0.009480
61	JOURNAL OF PHOTOCHEMISTRY AND PHOTOBIOLOGY B-BIOLOGY	17,015	6.252	0.012740

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
62	MOLECULAR ECOLOGY	44,625	6.185	0.040470
63	HUMAN MOLECULAR GENETICS	47,192	6.150	0.047520
64	BIOFACTORS	5,004	6.113	0.002810
65	Biomedicines	2,391	6.081	0.003650
66	JOURNAL OF NUTRITIONAL BIOCHEMISTRY	14,446	6.048	0.010580
67	INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES	139,463	5.923	0.195430
68	JOURNAL OF LIPID RESEARCH	28,376	5.922	0.019290
69	CELLULAR & MOLECULAR BIOLOGY LETTERS	2,216	5.787	0.002190
70	EXPERT REVIEWS IN MOLECULAR MEDICINE	2,042	5.600	0.000790
71	Reviews of Physiology Biochemistry and Pharmacology	865	5.545	0.000390
72	FEBS Journal	23,493	5.542	0.022540
73	Nucleic Acid Therapeutics	1,392	5.486	0.003070
74	JOURNAL OF MOLECULAR BIOLOGY	65,163	5.469	0.038400
75	BIOCHEMICAL SOCIETY TRANSACTIONS	14,862	5.407	0.014350
76	Food & Function	19,700	5.396	0.021550
77	BIOELECTROCHEMISTRY	6,107	5.373	0.004870
78	JOURNAL OF NEUROCHEMISTRY	40,281	5.372	0.019170
79	BIOORGANIC CHEMISTRY	10,576	5.275	0.010220
80	GENE THERAPY	8,112	5.250	0.004130
81	Frontiers in Molecular Biosciences	3,140	5.246	0.007660
82	CHROMOSOME RESEARCH	2,752	5.239	0.002250
83	CHEMICO-BIOLOGICAL INTERACTIONS	16,208	5.192	0.011950

Publikation 2:

Journal Data Filtered By: **Selected JCR Year: 2020** Selected Editions: SCIE,SSCI
 Selected Categories: **"PHARMACOLOGY and PHARMACY"** Selected Category
 Scheme: WoS

Gesamtanzahl: 275 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS DRUG DISCOVERY	41,989	84.694	0.048220
2	PHARMACOLOGICAL REVIEWS	14,442	25.468	0.009140
3	DRUG RESISTANCE UPDATES	4,315	18.500	0.004180
4	ADVANCED DRUG DELIVERY REVIEWS	43,769	15.470	0.028600
5	TRENDS IN PHARMACOLOGICAL SCIENCES	15,308	14.819	0.014880
6	Annual Review of Pharmacology and Toxicology	9,016	13.820	0.006590
7	MEDICINAL RESEARCH REVIEWS	6,835	12.944	0.005530
8	PHARMACOLOGY & THERAPEUTICS	21,235	12.310	0.021120
9	Acta Pharmaceutica Sinica B	6,314	11.413	0.000090
10	JOURNAL OF CONTROLLED RELEASE	60,586	9.776	0.044410
11	DRUGS	15,479	9.546	0.015960
12	BRITISH JOURNAL OF PHARMACOLOGY	42,870	8.739	0.026220
13	ALIMENTARY PHARMACOLOGY & THERAPEUTICS	25,641	8.171	0.031360
14	NEUROPSYCHOPHARMACOLOGY	30,856	7.853	0.034600
15	DRUG DISCOVERY TODAY	18,695	7.851	0.017400
16	PHARMACOLOGICAL RESEARCH	20,422	7.658	0.022420
17	Neurotherapeutics	6,764	7.620	0.009400
18	Current Neuropharmacology	6,080	7.363	0.007730
19	EXPERT OPINION ON THERAPEUTIC TARGETS	6,212	6.902	0.006470
20	CLINICAL PHARMACOLOGY & THERAPEUTICS	21,511	6.875	0.018360

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
21	EXPERT OPINION ON THERAPEUTIC PATENTS	4,196	6.674	0.004160
22	Expert Opinion on Drug Delivery	8,827	6.648	0.006290
23	European Heart Journal-Cardiovascular Pharmacotherapy	919	6.617	0.002480
24	Asian Journal of Pharmaceutical Sciences	2,993	6.598	0.003000
25	BIOMEDICINE & PHARMACOTHERAPY	41,024	6.529	0.056550
26	CLINICAL PHARMACOKINETICS	11,778	6.447	0.008390
27	DRUG DELIVERY	8,259	6.419	0.009000
28	International Journal of Nanomedicine	32,936	6.400	0.028680
29	Pharmaceutics	9,731	6.321	0.010160
30	EXPERT OPINION ON INVESTIGATIONAL DRUGS	5,964	6.206	0.005880
31	ACTA PHARMACOLOGICA SINICA	12,410	6.150	0.009060
32	Expert Opinion on Drug Discovery	4,902	6.098	0.006360
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35	ANTIVIRAL RESEARCH	15,686	5.970	0.018390
36	PHYTOTHERAPY RESEARCH	18,891	5.878	0.009070
37	INTERNATIONAL JOURNAL OF PHARMACEUTICS	61,118	5.875	0.031260
38	Pharmaceutics	5,462	5.863	0.005960
39	BIOCHEMICAL PHARMACOLOGY	33,633	5.858	0.017360
40	Frontiers in Pharmacology	34,494	5.810	0.055080
41	BIODRUGS	2,581	5.807	0.003770
42	JOURNAL OF ANTIMICROBIAL CHEMOTHERAPY	38,715	5.790	0.042490

Publikation 3:

Journal Data Filtered By: **Selected JCR Year: 2020** Selected Editions: SCIE,SSCI
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Gesamtanzahl: 108 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	npj Digital Medicine	2,406	11.653	0.007450
2	Implementation Science	14,750	7.327	0.020010
3	BMJ Quality & Safety	7,335	7.035	0.014360
4	ACADEMIC MEDICINE	22,828	6.893	0.027600
5	JOURNAL OF CLINICAL EPIDEMIOLOGY	36,224	6.437	0.028360
6	HEALTH AFFAIRS	22,442	6.301	0.047250
7	MEDICAL EDUCATION	14,132	6.251	0.011580
8	JOURNAL OF TELEMEDICINE AND TELE CARE	5,052	6.184	0.005600
9	VALUE IN HEALTH	12,642	5.725	0.017860
10	JOURNAL OF MEDICAL INTERNET RESEARCH	26,102	5.428	0.039100
11	JOURNAL OF GENERAL INTERNAL MEDICINE	26,727	5.128	0.028950
12	International Journal of Integrated Care	2,034	5.120	0.002800
13	International Journal of Health Policy and Management	2,118	5.007	0.005600
14	PHARMACOECONOMICS	6,543	4.981	0.009170
15	Journal of Personalized Medicine	1,071	4.945	0.002290
16	MILBANK QUARTERLY	4,632	4.911	0.005270
17	JMIR mHealth and uHealth	7,694	4.773	0.015520
18	PALLIATIVE MEDICINE	7,332	4.762	0.009100
19	BMC Medical Research Methodology	16,557	4.615	0.017550

Druckexemplare der Publikationen



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Molecular Sciences



Article

Anticancer Activity and Mechanisms of Action of New Chimeric EGFR/HDAC-Inhibitors

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Abstract: New chimeric inhibitors targeting the epidermal growth factor (EGFR) and histone deacetylases (HDACs) were synthesized and tested for antineoplastic efficiency in solid cancer (prostate and hepatocellular carcinoma) and leukemia/lymphoma cell models. The most promising compounds, 3BrQuin-SAHA and 3ClQuin-SAHA, showed strong inhibition of tumor cell growth at one-digit micromolar concentrations with IC₅₀ values similar to or lower than those of clinically established reference compounds SAHA and gefitinib. Target-specific EGFR and HDAC inhibition was demonstrated in cell-free kinase assays and Western blot analyses, while unspecific cytotoxic effects could not be observed in LDH release measurements. Proapoptotic formation of reactive oxygen species and caspase-3 activity induction in PCa and HCC cell lines DU145 and Hep-G2 seem to be further aspects of the modes of action. Antiangiogenic potency was recognized after applying the chimeric inhibitors on strongly vascularized chorioallantoic membranes of fertilized chicken eggs (CAM assay). The novel combination of two drug pharmacophores against the EGFR and HDACs in one single molecule was shown to have pronounced antineoplastic effects on tumor growth in both solid and leukemia/lymphoma cell models. The promising results merit further investigations to further decipher the underlying modes of action of the novel chimeric inhibitors and their suitability for new clinical approaches in tumor treatment.

Keywords: chimeric inhibitor; anticancer drugs; solid cancer; epidermal growth factor (EGFR); histone deacetylase (HDAC); anti-angiogenesis; prostate cancer; hepatoma; leukemia; lymphoma



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1. Introduction

In the last decades, a plethora of novel approaches for the treatment of advanced cancers has been developed. Among them, the concept of co-inhibiting distinct cellular tumor targets by so-called “chimeric inhibitors,” which merge two drug pharmacophores into a single molecule, has become a promising approach [1]. Attacking the tumor in dual or multiple ways is thought to hamper the occurrence or activation of tumor escape mechanisms known for single-mode chemotherapeutics and may help to diminish chemoresistance [2]. Compared with combination therapies, where different active compounds are given simultaneously or sequentially to address distinct cellular targets, the use of chimeric compounds offers several advantages and circumvents problems typical of combination therapies, such as different drug solubilities or physical incompatibilities which may lead to precipitation or drug inactivation. In addition, the risk of drug–drug interactions or the occurrence of concomitant adverse or unwanted side effects, which often require a complex dose adjustment, can be avoided when using chimeric inhibitors instead [1–3].

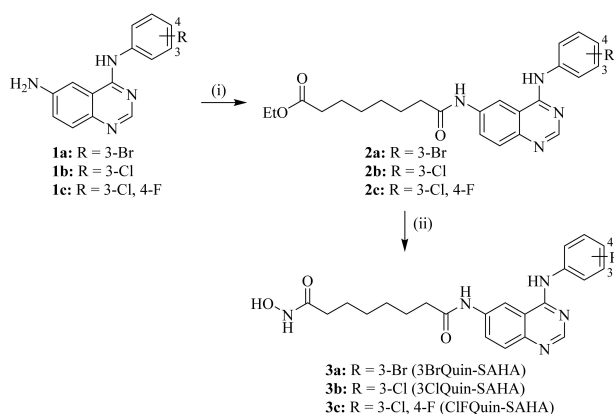
In the development of novel chimeric inhibitors, the hybridization of histone deacetylase inhibitors with receptor tyrosine kinase inhibitory pharmacophores has emerged as a particularly promising anticancer approach [4]. Histone deacetylases (HDACs), which are overexpressed in various cancers, are epigenetic regulators of chromatin condensation and decondensation. They have a strong impact on cancer cell proliferation, spreading, and metastasis. This renders HDAC inhibitors a promising novel class of compounds for targeted cancer therapy. Some HDAC inhibitors, such as the hydroxamic acid derivatives vorinostat or panobinostat, are already approved for the treatment of hematologic cancer diseases and are currently under intensive investigation for their use in solid tumors [5]. There are also drawbacks of the clinical application of single HDAC inhibitors, such as intrinsic or acquired drug resistance [4]. These may be overcome by merging an HDAC-inhibitory pharmacophore with another tumor-relevant inhibitor. In this respect, a pharmacophore directed against the epidermal growth factor receptor (EGFR) may be a promising hybridization partner, as the EGFR is also overexpressed and/or overactive in various solid tumors, including hepatocellular and prostate cancer [6,7]. Small molecule inhibitors of the EGFR and its tyrosine kinase activity, such as gefitinib, erlotinib, or lapatinib, are already clinically approved for the treatment of solid cancers [8,9].

In this paper, we report on the synthesis and biological evaluation of novel chimeric inhibitors which consist of newly designed tyrphostin derivatives functioning as epidermal growth factor inhibitors coupled with an HDAC-targeting hydroxamic acid moiety. HCC and PCa cell models were chosen as representatives of solid cancers and concomitant studies with lymphoma and leukemia cells were performed to underline the inhibitors relevance also for hematologic cancer diseases, thus indicating a broad spectrum of applicability.

2. Results

2.1. Chemistry

First, 1,8-Dioctanoic acid mono ethyl ester was reacted with the corresponding 6-aminoquinolines 1a–c and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) to afford ester precursors 2a–c (Scheme 1). Then, target compounds 3a–c were obtained from the reaction of 2a–c with aqueous hydroxylamine under basic conditions. Compounds 3a–c have a chimeric structure composed of an EGFR-targeting anilinoquinazoline moiety similar to approved EGFR inhibitors, such as gefitinib, and an oxoalkylhydroxamic acid appendage related to clinically applied HDAC inhibitors, such as SAHA, designed to interact with the Zn ions of the HDAC active sites.



Scheme 1. Reagents and conditions: (i) 1,8-dioctanoic acid mono ethyl ester, EDCI, DMAP, Et₃N, CH₂Cl₂, r.t., 24 h, 39–47%; (ii) 50% aq. HO-NH₂, NaOH, CH₂Cl₂/MeOH, r.t., 1 h, 43–64%.

2.2. Biological Evaluation

2.2.1. Antiproliferative Effects

The new chimeric compounds were investigated for their growth inhibitory effectiveness in human DU145 prostate cancer cells and human Hep-G2 hepatoblastoma cells (Table 1). Two compounds (3BrQuin-SAHA and 3ClQuin-SAHA) showed pronounced growth-inhibitory effects with IC_{50} values in the low micromolar range ($\sim 3\text{--}5\ \mu\text{M}$) in DU145 and Hep-G2 cells, that is, at concentrations far below those of the clinically relevant EGFR-inhibitor gefitinib, which was used as a reference for a single EGFR-inhibitor (IC_{50} of $\sim 12\ \mu\text{M}$ in DU145 cells and $>18\ \mu\text{M}$ in Hep-G2 cells). The IC_{50} value of SAHA, which served as clinically relevant reference for a single HDAC-inhibitor, amounted to $\sim 3\ \mu\text{M}$ in HepG2 cells, thus being in the range of those of 3BrQuin-SAHA and 3ClQuin-SAHA. In DU145 cells, SAHA was highly active, showing an IC_{50} value in the sub-micromolar range ($\sim 0.7\ \mu\text{M}$). Both 3BrQuin-SAHA and 3ClQuin-SAHA were further investigated for their time- and dose-dependent mode of action in DU145 and Hep-G2 cells (Figure 1a,b). Both 3BrQuin-SAHA and 3ClQuin-SAHA dose-dependently reduced the cell proliferation of DU145 and Hep-G2 cells significantly after only 24 h of treatment. The antiproliferative effects increased over time, and after 72 h, the maximum inhibition of prostate cancer DU145 cells amounted to $>95\%$ for both inhibitors. A comparable time- and dose-dependency was seen in hepatocellular Hep-G2 cancer cells with maximum inhibition of $\sim 80\%$ for 3BrQuin-SAHA and $\sim 85\%$ for 3ClQuin-SAHA after 72 h of treatment.

Table 1. Growth-inhibitory activity expressed as IC_{50} (μM) of novel chimeric inhibitors in human prostate cancer (DU145), hepatoma (Hep-G2), and human T-cell lymphoma cell lines, as determined after 48 h. SAHA and gefitinib were used as clinically relevant references for monomodal HDAC- or EGFR-inhibition, respectively. All results are described as means \pm SEM of $n \geq 3$ (for sensitive) or $n = 2$ (for insensitive compounds) independent experiments.

Compounds	DU145	Hep-G2	Jurkat	Hut78	SupT11	SMZ1
3BrQuin-SAHA (3a)	3.53 ± 0.23	4.94 ± 0.38	n.d.	n.d.	n.d.	n.d.
3ClQuin-SAHA (3b)	3.23 ± 0.18	3.92 ± 0.25	1.40 ± 0.12	1.18 ± 0.22	6.22 ± 0.25	2.24 ± 0.17
ClFQuin-SAHA (3c)	5.57 ± 0.38	>10	n.d.	n.d.	n.d.	n.d.
SAHA	0.68 ± 0.04	3.22 ± 0.44	1.7 ± 0.17	5.07 ± 0.42	4.67 ± 0.31	2.87 ± 0.33
Gefitinib	11.88 ± 2.13	18.53 ± 1.78	10.95 ± 0.28	>20	>20	>20

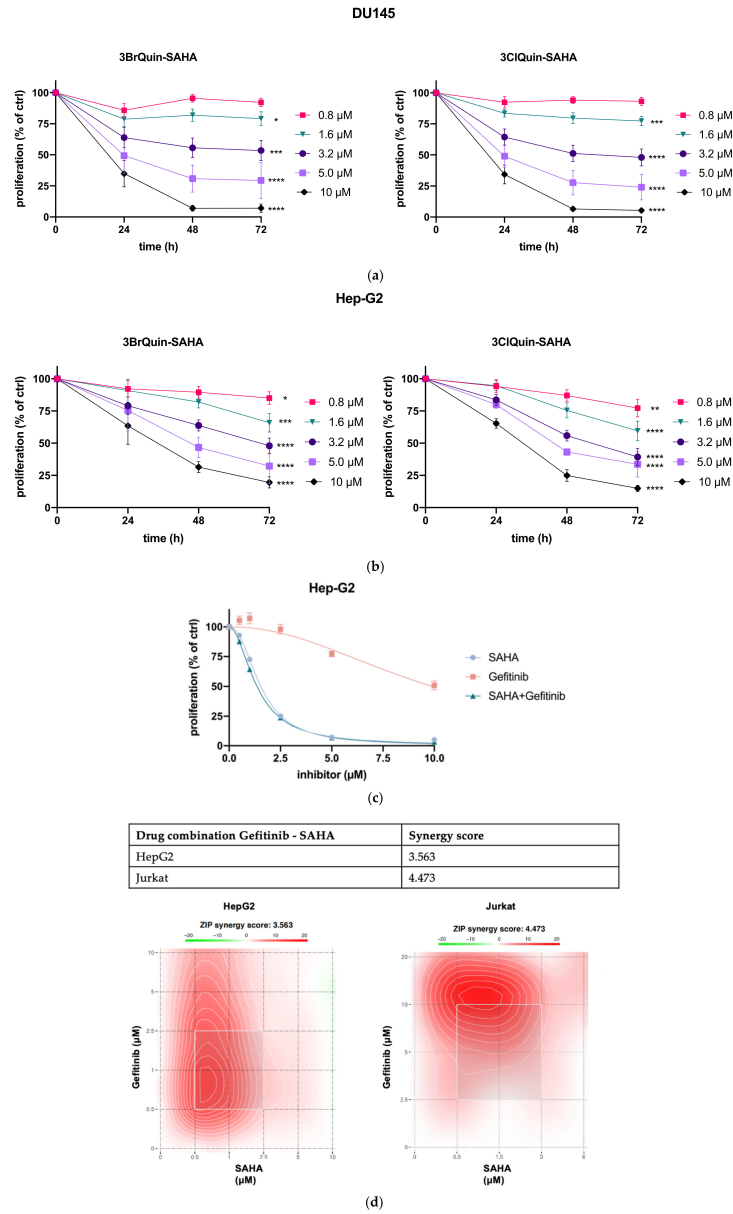


Figure 1. Growth-inhibitory effects of 3BrQuin-SAHA and 3CIQuin-SAHA in (a) PCa and (b) HCC cells. Time- and dose-dependent growth inhibition is given in percent, relative to untreated control cells, whose proliferation was set to 100%. (c) Curve shift experiment of the EGFR inhibitor gefitinib (red) and the HDAC inhibitor SAHA (blue) in a proliferation assay of the Hep-G2 cell line. (d) Calculation and visualization of synergy scores for drug combinations of Gefitinib and SAHA on Hep-G2 and Jurkat cells. Zero Interaction Potency (ZIP) synergy score calculated by Synergy Finder 2.0. A ZIP score from -10 to 10 describes that the interaction between two drugs is likely to be additive. Results are shown as means \pm SEM of $n \geq 3$ independent experiments. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$, **** $p \leq 0.0001$; linear regression.

The combination of SAHA and Gefitinib did not lead to synergistic antiproliferative effects in Hep-G2 and Jurkat cells (Figure 1c). Further analysis of the combination treatment employing SynergyFinder2.0 software underlined this finding. The calculated synergy scores of 3.563 for Hep-G2 point towards an additive rather than a synergistic effect (Figure 1d).

Unlike for solid tumors, single HDAC inhibitors such as SAHA or panobinostat are either already approved for the treatment of hematologic neoplasms such as T-cell leukemias/lymphomas or show promising pre-clinical data [10]. Nevertheless, the combination of kinase inhibition with HDAC inhibition has the potential to achieve significant improvements here, too. Hence, 3CIQuin-SAHA, which was the most active compound in the tested solid tumor cell models, was also selected for tests concerning its activity against a panel of lymphoma and leukemia cell lines (Jurkat, Hut78, SupT11, and SMZ1). The IC_{50} values of 3CIQuin-SAHA were in the low single-digit μ M concentration range for all four cell lines (Table 1). Interestingly, 3CIQuin-SAHA was slightly more active than the SAHA, which served as a reference for a clinically approved HDAC-inhibitor. Likewise, the EGFR-inhibitor gefitinib showed a markedly less pronounced antiproliferative efficacy in Jurkat cells, and no appreciable effect in the other three T-cell lymphoma cell lines, as compared with 3CIQuin-SAHA. The novel chimeric inhibitor 3CIQuin-SAHA was shown to have pronounced antiproliferative potency also in hematologic tumor cell models. However, the enhanced antiproliferative effect of the 3CIQuin-SAHA in T-cell lymphoma cells may not be easily attributed to an additive or even synergistic effect of the HDAC-and EGFR-inhibiting pharmacophores of the compound, because additional FACS analyses on the expression of EGFR receptors in the T-cell lymphoma cell lines revealed that none of the four cell lines expressed EGFR receptors on their cell surface (Supplementary Figure S1). Not surprisingly, the combination treatment of Jurkat cells with SAHA and Gefitinib did not lead to synergistic or pronounced additive antiproliferative effects, but rather reflected the effect of SAHA (Figure 1d). Further investigations are needed to clarify the underlying mode of action of 3CIQuin-SAHA in lymphoma cells. Nevertheless, at this stage, the very fact that the novel chimeric inhibitors exert antiproliferative effects which are in the range of the clinically relevant monomodal HDAC-inhibitor SAHA underlines their broad efficacy.

Hence, the novel chimeric inhibitor 3CIQuin-SAHA was shown to have superior antiproliferative potency also in hematologic tumor cell models. However, the enhanced antiproliferative effect of the 3CIQuin-SAHA in T-cell lymphoma cells may not be easily attributed to an additive or even synergistic effect of the HDAC-and EGFR-inhibiting pharmacophores of the compound, because additional FACS analyses on the expression of EGFR receptors in the T-cell lymphoma cell lines revealed that none of the four cell lines expressed EGFR receptors on their cell surface (Supplementary Figure S1). Thus, further investigations are needed to clarify the underlying mode of action of 3CIQuin-SAHA in lymphoma cells. Nevertheless, at this stage, the very fact that the novel chimeric inhibitors exceeded the antiproliferative effects of the clinically relevant monomodal HDAC-inhibitor SAHA underlines their broad efficacy.

2.2.2. Cytotoxicity of 3BrQuin-SAHA and 3CIQuin-SAHA

To check for cytotoxic effects possibly contributing to the antiproliferative effects of 3BrQuin-SAHA and 3CIQuin-SAHA treatment, the release of lactate dehydrogenase (LDH) from the cytosol into the supernatant of DU145 and Hep-G2 cell cultures was measured. Increased LDH release indicates necrotic cell death due to a treatment-induced damage of cell membranes [11]. However, neither 3BrQuin-SAHA nor 3CIQuin-SAHA induced significant increases in LDH release after 3 and 24 h of treatment with rising compound concentrations (1–10 μ M), but rather showed a nonsignificant oscillation of $\pm 5\%$ around the basal LDH release of untreated controls (Figure 2). The data indicate that even at high concentrations, both compounds do not affect cell membrane integrity. Thus, an induction of immediate cytotoxicity is unlikely to account for the observed antiproliferative effects of the novel inhibitors. Moreover, as expected for SAHA and gefitinib, which were used

as monomodal HDAC- and EGFR-inhibitor references, no relevant induction of necrosis was observed. Likewise, the combination of SAHA and gefitinib in Hep-G2 cells did not exhibit increased cytotoxicity either. (Figure 2b).

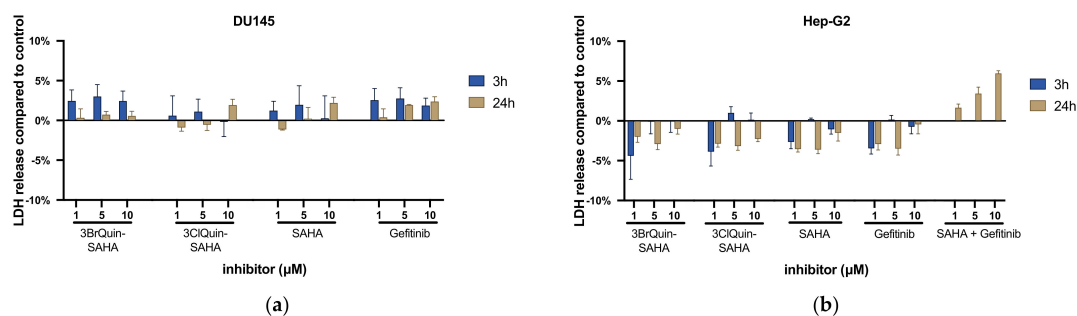


Figure 2. Unspecific cytotoxic effects of 3BrQuin-SAHA and 3CIQuin-SAHA in (a) prostate cancer and (b) hepatoma cells. LDH release of DU145 and Hep-G2 cells was measured after 3 and 24 h of incubation with 1, 5, or 10 μM of 3BrQuin-SAHA or 3CI-Quin-SAHA, as well as SAHA and gefitinib alone or in combination on Hep-G2 cells. Results show changes in LDH release relative to untreated controls. Data are given as percentage changes relative to basal LDH release of controls. Means \pm SEM of $n = 3$ independent experiments.

2.2.3. EGFR Tyrosine Kinase Inhibition by 3BrQuin-SAHA and 3CIQuin-SAHA

Both 3BrQuin-SAHA and 3CIQuin-SAHA were tested for their EGFR tyrosine kinase inhibition by performing a luminescence-based, cell-free EGFR kinase activity assay. As shown in Figure 3a, a pronounced and dose-dependent inhibition of EGFR kinase activity of up to ~75% was observed upon treatment with the novel chimeric inhibitors. As expected, the clinically established EGFR-inhibitor gefitinib, used as a positive control, also showed a pronounced effectiveness and inhibited EGFR kinase activity up to ~85%. We also checked for cellular effects of the novel compounds by determination of changes in the protein level of the EGFR in DU145 prostate cancer cells. Western blots for the EGFR expression level of treated vs. nontreated DU145 cells revealed that 3BrQuin-SAHA and, even more so, 3CIQuin-SAHA led to a significant downregulation of EGFR protein (Figure 3b,c). The effect of both chimeric inhibitors was much more pronounced than that of gefitinib, and thus we wondered whether the HDAC-inhibitory moiety of the chimeric inhibitors may have an inhibitory effect on the EGFR protein expression. Indeed, treatment of DU145 cells with SAHA alone led to a pronounced and significant suppression in the expression of the EGFR that even exceeded that of gefitinib. Thus, we could show that the tyrophostin-like pharmacophore of the novel compounds is responsible for an effective inhibition of the EGFR-TK-activity and that the hydroxamate pharmacophore contributes to a significant suppression of the expression of the EGFR, which may lead to a synergistic or at least additional overall efficacy against EGFR-mediated cellular effects.

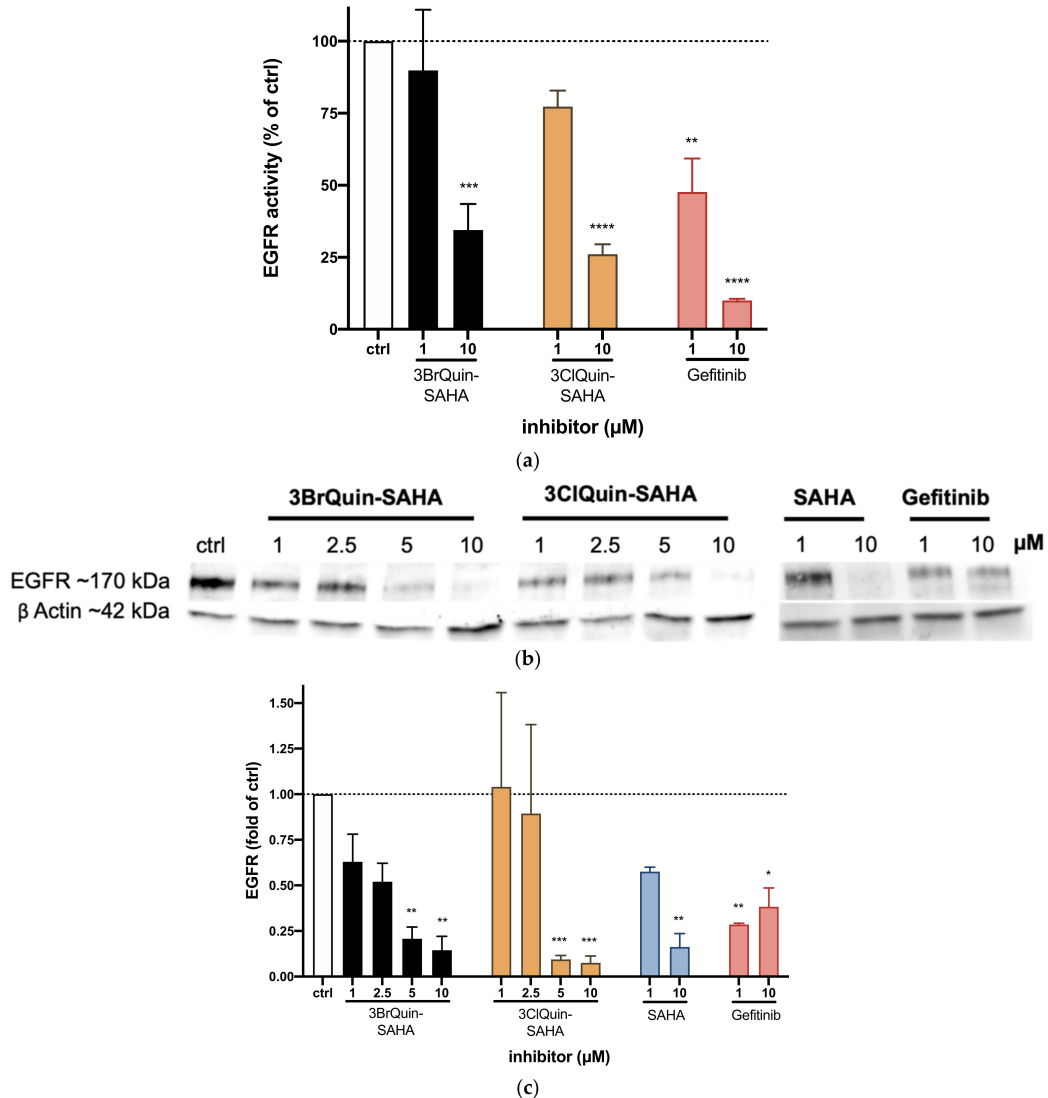


Figure 3. Inhibitory effects of 3BrQuin-SAHA and 3CIQuin-SAHA on EGFR tyrosine kinase activity and protein expression. (a) Dose-dependent inhibition of EGFR kinase activity by the novel chimeric inhibitors and gefitinib was determined in a cell-free EGFR-kinase assay. Means \pm SEM of three independent experiments showing the percentage inhibition of treated vs. untreated controls whose kinase activity was set to 100%. (b) Representative Western blots of $n = 3$ independent experiments showing treatment induced changes in the expression of EGFR in DU145 cells after 24 h. β -actin was used as loading control. (c) Out of these Western blots' gray intensity, mean \pm SEM of EGFRs' specific bands were quantified and adjusted to protein-loading and normalized to untreated control. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$, **** $p \leq 0.0001$; two-way ANOVA Dunnett's post hoc test.

2.2.4. HDAC Inhibition by 3BrQuin-SAHA and 3CIQuin-SAHA

To assess the HDAC-inhibitory potency of the novel dual-mode hydroxamic acids, commercially available HeLa cell nuclear extracts, rich in HDAC activity and constituting a cell-free pan-HDAC enzyme profile, were treated with 3BrQuin-SAHA and 3CIQuin-SAHA (1–10 μM). Both compounds strongly inhibited the HDAC activity in a dose-dependent manner, leading to IC_{50} values in the sub-micromolar range with 302 nM (for 3BrQuin-SAHA) and 474 nM (for 3CIQuin-SAHA), respectively. Compared with the hydroxamic acid SAHA ($\text{IC}_{50} = 20$ nM), the mainstay of HDAC-targeting anticancer therapy, the pan-HDAC-inhibitory potency of the novel compounds is distinctly weaker, yet still in a clinically meaningful range (Figure 4a) [12]. Their HDAC-inhibitory efficacy was further evaluated in prostate cancer cells by immunodetection of the increased portion of acetylated histone H3, which is part of the cellular nucleosome. Western blot analysis revealed a dose-dependent rise of H3 acetylation of DU145 cells upon treatment with 3BrQuin-SAHA and 3CIQuin-SAHA or SAHA as a consequence of the suppression of the histone deacetylating activity of HDACs (Figure 4b,c).

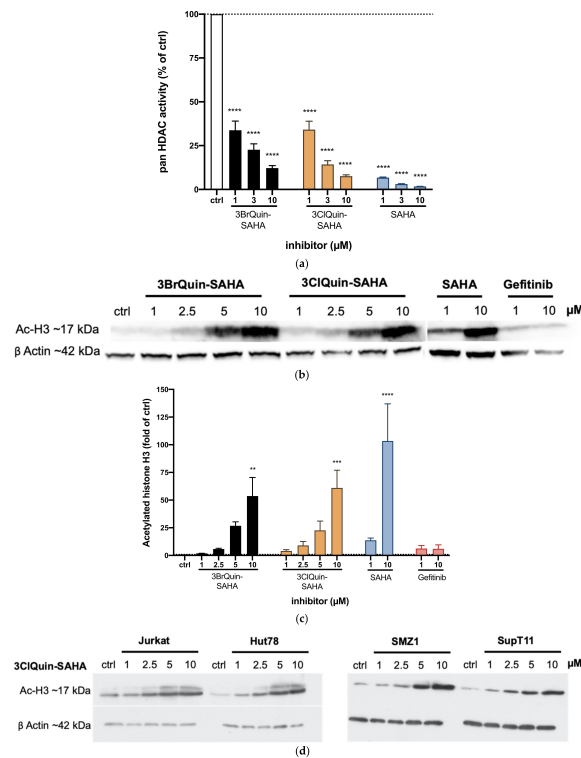


Figure 4. HDAC inhibitory effects of 3BrQuin-SAHA and 3CIQuin-SAHA. (a) HDAC activity was measured by luminescence display of the acetylation state of histones. HeLa nuclear cell extracts, used as source for histones, were preincubated with substrate and inhibitor. Results are given as relatives to controls, not preincubated with inhibitor, as means \pm SEM of $n = 3$ independent experiment. (b) Representative Western blot out of $n = 3$ experiments, showing changes in the expression of acetylated histone H3 after 24 h treatment with compounds in DU145 cells. (c) Out of the DU145 Western blots' gray intensity, mean \pm SEM of acetylated histone-H3-specific bands were quantified, adjusted to protein-loading, and normalized to untreated control. (d) Western blots, showing changes in the expression of acetylated histone H3 after 24 h treatment with 3CIQuin-SAHA in Jurkat, Hut78, SupT11, and SMZ1 human T-cell leukemia/lymphoma cell lines. ** $p \leq 0.005$, *** $p \leq 0.0005$, **** $p \leq 0.0001$; two-way ANOVA Dunnett's post hoc test.

Increased acetylation of histone H3 upon treatment with 3CIQuin-SAHA was also seen in the four T-cell lymphoma cell lines (Figure 4d), confirming the HDAC-inhibitory activity of the compound also for the hematologic cancer cell models.

2.2.5. Subtype Specific HDAC Inhibition of 3BrQuin-SAHA and 3CIQuin-SAHA

Both 3BrQuin-SAHA and 3CIQuin-SAHA were also tested for HDAC-subtype-specific inhibition using recombinant HDAC 2 and 6 enzymes. Both HDACs showed high expression levels in untreated DU145 cells (Figure 5a). The activity of HDAC 6, interesting because of its cytosolic localization and chaperone modulating function, was shown to be strongly inhibited in a dose-dependent manner when treated with 1–10 μM of the novel chimeric compounds (Figure 5b) [13]. Similar effects were found for HDAC 2 (Figure 5c), a class 1 HDAC highly overexpressed in PCa and HCC cells [14,15].

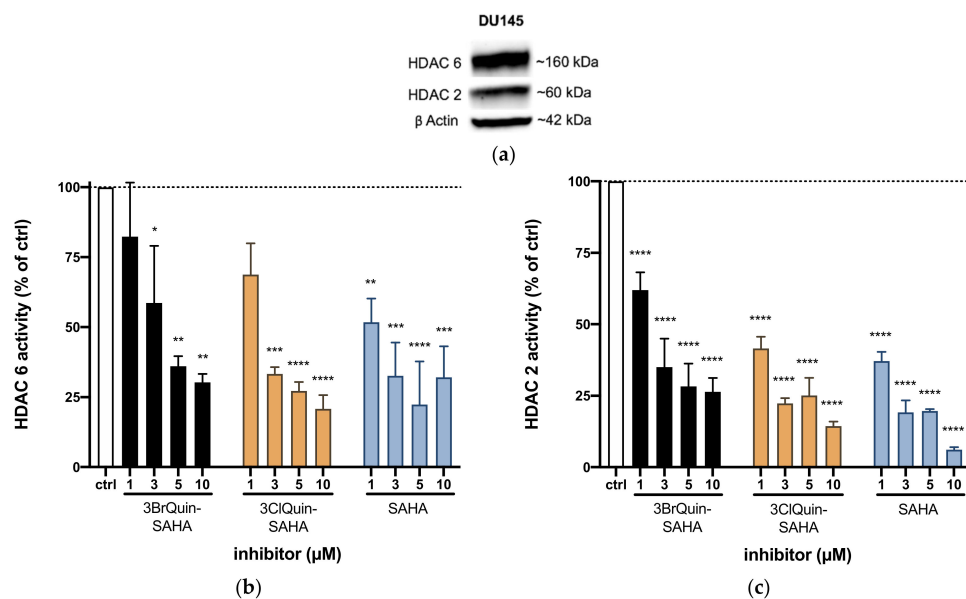


Figure 5. HDAC subtype specific inhibitory effects of 3BrQuin-SAHA and 3CIQuin-SAHA. (a) Western blot showing the expression levels of HDAC 2 and HDAC 6 in untreated DU145 cells (ctrl = control). (b) Dose-dependent inhibition of HDAC 6 and HDAC 2 (c) activity by 3BrQuin-SAHA, 3CIQuin-SAHA, or SAHA, respectively. Results, relative to untreated controls, as means \pm SEM of $n = 3$ independent experiments. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$, **** $p \leq 0.0001$; two-way ANOVA Dunnett's post hoc test.

2.2.6. Induction of ROS and Apoptosis by 3BrQuin-SAHA and 3CIQuin-SAHA

For a deeper insight into the molecular events underlying the mode of action of the novel chimeric inhibitors, we investigated their possible involvement in the induction of the formation of reactive oxygen species (ROS), since HDAC inhibition has already been shown to be linked to ROS induction in solid cancers, including prostate cancer [16]. Treatment of DU145 and Hep-G2 cells with 3BrQuin-SAHA and 3CIQuin-SAHA led to a pronounced time- and dose-dependent increase in cytosolic ROS after 24 h, as evidenced by fluorescence microscopy with the cytosol-specific ROS dye CellROX orange. Notably, a similar treatment with equimolar concentrations of SAHA or gefitinib elicited a much weaker (SAHA) or almost no (gefitinib) ROS increase in prostate and hepatocellular cancer cells (Figure 6a,b).

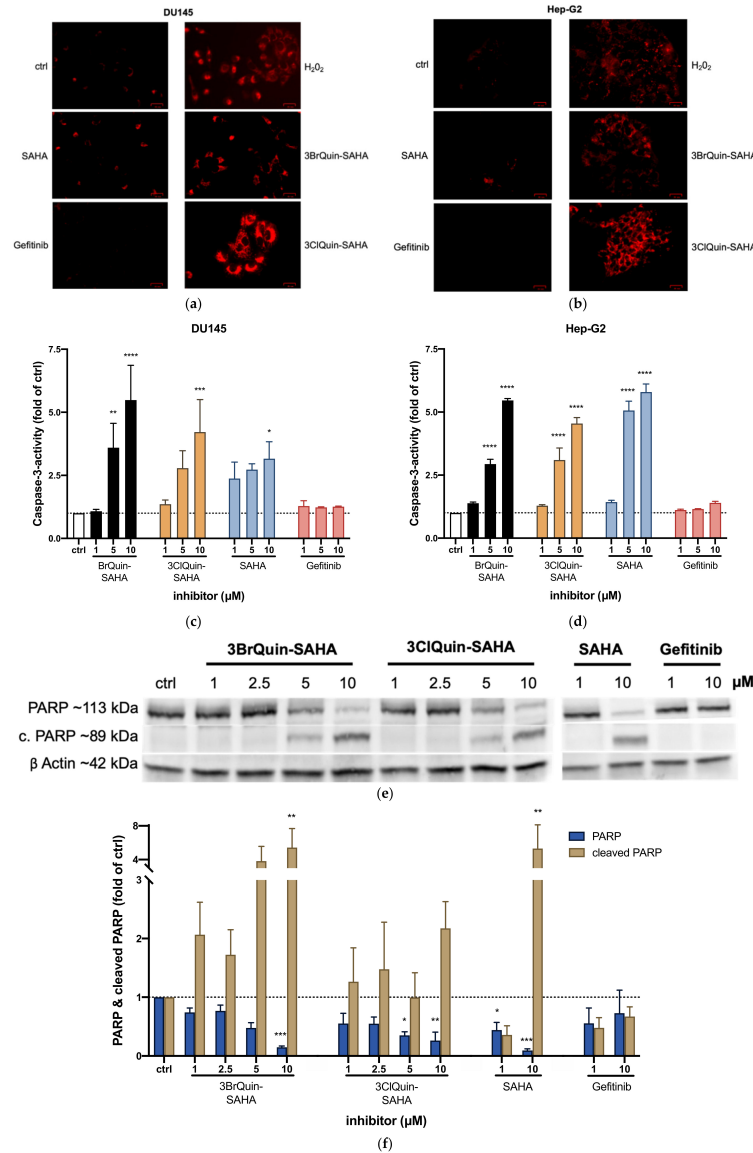


Figure 6. Apoptosis induction by 3BrQuin-SAHA and 3CIQuin-SAHA. ROS induction after 24 h of incubation with 10 μM 3BrQuin-SAHA, 3CIQuin-SAHA, SAHA, and gefitinib in DU145 (a) and for Hep-G2 cells (b). H₂O₂ (1.6 mM) was used as positive control. Representative findings out of *n* = 3 independent experiments. Caspase-3 induction in DU145 (c) or Hep-G2 cells (d) after 24 h treatment with 3BrQuin-SAHA, 3CIQuin-SAHA, SAHA, and gefitinib. Means ± SEM of *n* = 3 independent experiments. (e) Representative Western blot out of *n* = 3 experiments, showing changes in the expression PARP and PARP after cleavage in DU145 cells after 24 h treatment with compound. (f) Out of these Western blots' gray intensities, PARP- and cleaved-PARP-specific bands were quantified, adjusted to protein-loading, and normalized to untreated controls. Data are given as means ± SEM of *n* = 3 independent experiments. * *p* ≤ 0.05, ** *p* ≤ 0.005, *** *p* ≤ 0.0005, **** *p* ≤ 0.0001; two-way ANOVA Dunnett's post hoc test.

ROS induction has been linked to the activation of caspases and may thus serve as a trigger of apoptosis [17]. To evaluate if apoptosis may play a role in the antiproliferative effects of the novel chimeric inhibitors, we checked them for the activation of the apoptosis-specific effector caspase-3. Upon treatment with 3BrQuin-SAHA and 3ClQuin-SAHA, a pronounced time- and dose-dependent caspase-3 activation became apparent. The onset of caspase-3 activation was visible after 6 h of treatment with 10 μ M (Figure 6c,d). Treatment with SAHA elicited comparable caspase-3 inductions, whereas virtually no induction of caspase-3-activity was seen with gefitinib. Further Western blot analyses revealed a concomitant induction of apoptosis-specific cleavage of poly(ADP-ribose)-polymerase (PARP) (Figure 6e,f). PARP cleavage to its smaller ~85 kDa fragment is known to be caspase-3-driven and may be used as a marker for chemotherapy-induced apoptosis [18,19]. Our findings contribute to the assumption that apoptosis may play a prominent role for the effects that we observed upon treatment of Hep-G2 and DU145 cells with 3BrQuin-SAHA and 3ClQuin-SAHA.

2.2.7. In Vivo Assessment of Antiangiogenic Effects of 3BrQuin-SAHA and 3ClQuin-SAHA on the CAM of Fertilized Chicken Eggs

Finally, the chimeric dual-mode inhibitors were screened for antiangiogenic effects in a systemic scenario by investigating treatment-induced changes in the vasculature of the chorioallantoic membrane (CAM) of fertilized chicken eggs (CAM assay) [20]. Qualitative comparisons between untreated (PBS) areas of the CAM to those treated with 3BrQuin-SAHA or 3ClQuin-SAHA showed a reduction of micro-vessels (Figure 7, blue arrows) after 72 h with the latter. Furthermore, respective single blood vessels displayed morphological irregularities such as blunted ends (Figure 7, red arrow). These features seemed to be more prominent in eggs treated with 3ClQuin-SAHA compared to those treated with 3BrQuin-SAHA. The monomodal reference compounds SAHA and gefitinib also showed antiangiogenic effects, which were, however, not as prominent as the ones caused by 3ClQuin-SAHA. Additionally, it is worth noting that there was no higher embryonic lethality observed in eggs treated with the compounds, which supports the notion from the LDH-release assays (Section 2.2.3; Figure 2) that the novel compounds do not exert general cytotoxic effects.

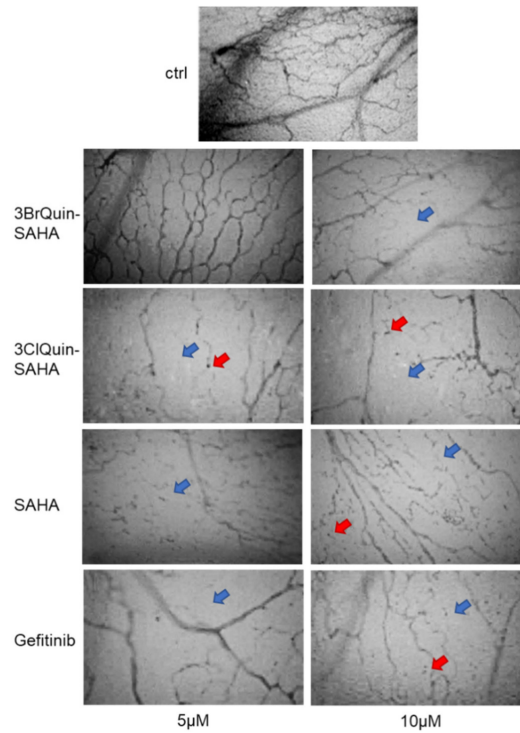


Figure 7. Antiangiogenic effects of 3BrQuin-SAHA and 3CIQuin-SAHA. Representative images from $n = 3$ preparations per condition of treatment-induced changes in the development and morphology of the vasculature of the chorioallantoic membrane (CAM) of fertilized chicken eggs after 72 h of topical treatment with 3BrQuin-SAHA, 3CIQuin-SAHA, SAHA, and gefitinib. Compared to untreated control CAMs, a dose-dependent reduction in the number (blue arrow) and altered morphology (red arrow) of CAM micro-vessels was observed. Most pronounced effects were seen in 3CIQuin-SAHA-treated CAMs.

3. Discussion

New tumor therapies for HCC and PCa are desperately needed due to poor treatment alternatives for patients with advanced tumor progression states. Resistance to first-line therapies is a common phenomenon and a major drawback for an efficient treatment [21,22]. As reported earlier, the overexpression of HDACs and EGFR is reported for tumors of different entities, including PCa and HCC [6,7,14,15].

In this study, we introduced new dual inhibitors combining tyrosinase AG1478- and indazole-derivates with a hydroxamic acid and addressing both EGFR and HDAC activities.

Increased cell proliferation and compensatory inhibition of apoptosis are critical events that are required for the growth and development of cancer [23]. Targeting these key events is important for potent and specific anticancer therapies. We found that the two novel chimeric inhibitors 3BrQuin-SAHA and 3CIQuin-SAHA effectively inhibited the growth of PCa (DU145) and HCC (HepG2) cells at low micromolar concentrations, with IC_{50} (48 h) values between ~ 3 – $5 \mu\text{M}$.

There are several studies which showed that targeting the HDAC activity in different cancer cells in combination with EGFR inhibition is a promising approach [24]. Bruzzese et al. demonstrated that the monomodal HDAC inhibitor SAHA in combination with the EGFR tyrosine kinase inhibitor gefitinib induced a synergistic inhibition of proliferation,

migration, and invasion, as well as induction of apoptosis in squamous cell carcinoma of head and neck cells, including cells resistant to gefitinib [25]. Additional studies showed that the combination of these two drugs exhibited a high anticancer activity in various tumor entities [26,27]. The chimeric dual kinase/HDAC inhibitor CUDC-101 has already undergone clinical trials in patients with various solid tumors. The results were very promising, underlining the high relevance of this compound class [4].

To better understand the specific modes of action, the apoptosis induction via caspase-3-activity and PARP cleavage was investigated. It was found that 3BrQuin-SAHA, 3ClQuin-SAHA, as well as SAHA led to a prolonged activation of caspase-3 activity with a maximum of induction after 24 h of treatment. Concomitant to caspase-3 activation, PARP depletion due to its cleavage by caspase was observed in Western blot analyses. All these findings indicate that 3BrQuin-SAHA and 3ClQuin-SAHA are potent inducers of apoptosis in HCC and PCa cells. Their EGFR- and HDAC-inhibitory potency were proven in enzyme assays and Western blot analyses and confirmed by similar effects on purified HDAC2 and HDAC6 enzymes.

The expression of HDACs is upregulated in HCC and prostate cancer, and this up-regulation is correlated with patient survival [14,15]. HDAC 2 expression was shown to be necessary for apoptosis prevention, mirrored by the finding that HDAC 2 inhibition increased apoptosis in colon carcinoma cells [28,29]. Quint et al. introduced HDAC 2 up-regulation as a predictor for survival of HCC patients [30]. Several studies showed that HDAC inhibition by SAHA leads to higher apoptosis also in PCa tumor cells, independently of a specific HDAC subtype [31,32]. These findings are in line with our measurements showing high apoptosis rates in HCC and PCa cancer cells after treatment with SAHA but not with gefitinib.

Interestingly, both new chimeric inhibitors as well as SAHA downregulated the expression of the EGFR in DU145 cells, indicating that the HDAC inhibitory moieties of the novel chimeric inhibitors may also affect the expression of EGFR. In conjunction with the findings on the regulation of EGFR activity in the cell-free EGFR kinase assays, our data indicate a dual activity of the novel compounds towards EGFR expression and downregulation of EGFR kinase activity. This would explain the enhanced overall anti-EGFR effect of the chimeric compounds compared to that of gefitinib alone. This could also be interesting for appraising expected/unexpected side effects, as well as therapeutic efficacy and therapy resistance [4].

In a first systemic evaluation employing CAM assays in which the vasculature of the chorioallantoic membrane of fertilized chicken eggs served as a substrate for the determination of antiangiogenic effects, we could finally show that 3BrQuin-SAHA and 3ClQuin-SAHA also exerted antiangiogenic effects on the vascular network of the CAM, while no appreciable cytotoxic side effects were observed, since the survival of the developing chicken embryos was not affected by the treatment.

With this work, we shed first light on the modes of action of the two novel chimeric inhibitors 3ClQuin-SAHA and 3BrQuin-SAHA, combining HDAC- and EGFR-inhibiting moieties in one molecule. The compounds were found to encompass pronounced anti-proliferative, apoptosis-inducing, and anti-angiogenic effects in solid tumor cell models, warranting further studies to decipher in more depth the underlying modes of action and suitability of these promising representatives of the rapidly emerging class of chimeric inhibitors as candidates for future cancer treatment.

4. Materials and Methods

4.1. General Procedures and Materials

Chemicals and reagents were purchased from Sigma-Aldrich. Compounds 1a-c were prepared according to literature procedures [33,34]. The following instruments were used: determination of melting points (uncorrected), Gallenkamp; IR spectra generation, Perkin-Elmer Spectrum One FT-IR spectrophotometer with ATR sampling unit; obtaining nuclear magnetic resonance spectra (NMR), BRUKER Avance 300 spectrometer; chemical shifts

are given in parts per million (δ) downfield from tetramethylsilane as internal standard; generating mass spectra (MS), Varian MAT 311A (EI); Thermo Fischer Scientific Q Exactive (ESI-HRMS); microanalyses, Elementar UNICUBE (Elementar Analysensysteme GmbH).

4.2. Synthesis of Novel Chimeric HDAC/EGFR Inhibitors

Ethyl-8-[4-(3-bromophenylamino)quinazolin-6-ylamino]-8-oxooctanoate (2a, 3BrQuin-SAHEt)

4-(3-Bromophenylamino)-6-aminoquinazoline (80 mg, 0.254 mmol) was dissolved in dry CH_2Cl_2 and ethyl hydrogen suberate (50 mg, 0.254 mmol), EDCI (75 mg, 0.39 mmol), DMAP (15 mg, 0.10 mmol), and triethyl amine (182 μL , 0.83 mmol) were added. After stirring at room temperature for 24 h, the solvent was evaporated, and the residue was purified by column chromatography (silica gel 60). Yield: 58 mg (0.12 mmol, 47%); $R_f = 0.42$ (ethyl acetate); colorless solid of m.p. 163–164 $^\circ\text{C}$; $\nu_{\text{max}}(\text{ATR})/\text{cm}^{-1}$ 3286, 3099, 2931, 2852, 1733, 1667, 1627, 1593, 1572, 1526, 1476, 1418, 1392, 1381, 1357, 1327, 1290, 1242, 1226, 1176, 1132, 1090, 1072, 1062, 1030, 997, 974, 960, 949, 915, 869, 852, 843, 791, 777, 709, 681; ^1H NMR (300 MHz, DMSO-d_6) δ 1.17 (3 H, t, $J = 7.1$ Hz), 1.3–1.4 (4 H, m), 1.5–1.7 (4 H, m), 2.2–2.3 (2 H, m), 2.39 (2 H, t, $J = 7.4$ Hz), 4.05 (2 H, q, $J = 7.1$ Hz), 7.2–7.4 (2 H, m), 7.7–7.9 (3 H, m), 8.17 (1 H, s), 8.57 (1 H, s), 8.73 (1 H, s), 9.91 (1 H, s), 10.25 (1 H, s); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 14.1, 24.3, 24.8, 28.2, 28.3, 33.4, 36.1, 59.6, 111.6, 115.4, 120.8, 121.1, 124.3, 125.8, 127.1, 128.4, 130.3, 137.0, 141.2, 146.5, 152.9, 157.2, 171.4, 172.9; m/z (%) 500 (84) $[\text{M}^+]$, 498 (84) $[\text{M}^+]$, 455 (13), 453 (21), 315 (100), 234 (26), 83 (23), 69 (23), 55 (27), 41 (22).

N-[4-(3-Bromophenylamino)quinazolin-6-yl]-N-hydroxyoctanediamide (3a, 3BrQuin-SAHA)

Ethyl-8-[4-(3-bromophenylamino)quinazolin-6-ylamino]-8-oxooctanoate (54 mg, 0.11 mmol) was dissolved in $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (9 mL, 1:2); then, hydroxylamine (50% in water, 0.5 mL, 15 mmol) and NaOH (200 mg, 5 mmol) were added, and the reaction mixture was stirred at room temperature for 1 h. The solvent was removed, and the residue was dissolved in water and adjusted to pH 8 with acetic acid. The aqueous phase was extracted with ethyl acetate (2 \times 50 mL) with a small amount of MeOH, dried over Na_2SO_4 and concentrated in vacuum. The solid residue was recrystallized from $\text{CH}_2\text{Cl}_2/n$ -hexane, washed with water in order to remove acetate impurities, and dried in vacuum. Yield: 58 mg (0.12 mmol, 47%); colorless solid of m.p. 241–242 $^\circ\text{C}$; $\nu_{\text{max}}(\text{ATR})/\text{cm}^{-1}$ 3226, 2924, 2853, 1665, 1622, 1574, 1525, 1474, 1426, 1388, 1366, 1330, 1238, 1138, 1099, 1058, 981, 954, 925, 879, 840, 769, 723, 668; ^1H NMR (300 MHz, DMSO-d_6) δ 1.2–1.4 (4 H, m), 1.5–1.6 (2 H, m), 1.6–1.7 (2 H, m), 1.95 (2 H, t, $J = 7.3$ Hz), 2.38 (2 H, t, $J = 7.3$ Hz), 7.2–7.4 (2 H, m), 7.7–7.9 (3 H, m), 8.17 (1 H, s), 8.57 (1 H, s), 8.67 (1 H, s), 8.74 (1 H, s), 9.91 (1 H, s), 10.25 (1 H, s), 10.36 (1 H, s); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 24.9, 25.0, 28.4, 32.2, 36.2, 111.6, 115.5, 120.8, 121.1, 124.2, 125.8, 127.2, 128.4, 130.3, 137.0, 141.2, 152.9, 157.2, 171.4; m/z (ESI, %) 487.8 (83) $[\text{M}^+]$, 485.8 (85) $[\text{M}^+]$, 338.1 (100); Anal. calcd. ($\text{C}_{22}\text{H}_{24}\text{BrN}_5\text{O}_3$) C, 54.33; H, 4.97; N, 14.40; Found C, 54.10; H, 4.91; N, 14.29.

Ethyl-8-[4-(3-chlorophenylamino)quinazolin-6-ylamino]-8-oxooctanoate (2b, 3ClQuin-SAHEt)

4-(3-Chlorophenylamino)-6-aminoquinazoline (120 mg, 0.44 mmol) was dissolved in dry CH_2Cl_2 and ethyl hydrogen suberate (90 mg, 0.44 mmol), and EDCI (130 mg, 0.68 mmol), DMAP (26 mg, 0.174 mmol), and triethyl amine (315 μL , 1.43 mmol) were added. After stirring at room temperature for 24 h, the solvent was evaporated, and the residue was purified by column chromatography (silica gel 60). Yield: 76 mg (0.17 mmol, 39%); $R_f = 0.38$ (ethyl acetate); colorless solid of m.p. 159–160 $^\circ\text{C}$; $\nu_{\text{max}}(\text{ATR})/\text{cm}^{-1}$ 3286, 3100, 2930, 2852, 1733, 1667, 1628, 1598, 1573, 1526, 1479, 1467, 1419, 1381, 1392, 1357, 1327, 1290, 1227, 1190, 1176, 1096, 1075, 1064, 1027, 999, 974, 951, 916, 880, 861, 843, 800, 791, 780, 710, 684; ^1H NMR (300 MHz, DMSO-d_6) δ 1.17 (3 H, t, $J = 7.1$ Hz), 1.3–1.4 (4 H, m), 1.5–1.7 (4 H, m), 2.2–2.3 (2 H, m), 2.39 (2 H, t, $J = 7.4$ Hz), 4.04 (2 H, q, $J = 7.1$ Hz), 7.1–7.2 (1 H, m), 7.3–7.4 (1 H, m), 7.7–7.9 (3 H, m), 8.04 (1 H, s), 8.57 (1 H, s), 8.72 (1 H, s), 9.89 (1 H, s), 10.22 (1 H, s); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 14.1, 24.3, 24.8, 28.2, 28.3, 33.4, 36.1, 59.6, 111.6, 115.4, 120.4, 121.4, 122.9, 127.1,

128.4, 129.9, 132.6, 137.0, 141.1, 146.5, 152.9, 157.2, 171.4, 172.8; m/z (%) 454 (93) [M⁺], 409 (21), 269 (100).

N-[4-(3-Chlorophenylamino)quinazolin-6-yl]-N-hydroxyoctanediamide (3b, 3ClQuin-SAHA)

Ethyl-8-[4-(3-chlorophenylamino)quinazolin-6-ylamino]-8-oxooctanoate (74 mg, 0.16 mmol) was dissolved in CH₂Cl₂/MeOH (9 mL, 1:2). Hydroxylamine (50% in water, 0.5 mL, 15 mmol) and NaOH (200 mg, 5 mmol) were added, and the reaction mixture was stirred at room temperature for 1 h. The solvent was removed, and the residue was dissolved in water and adjusted to pH 8 with acetic acid. The aqueous phase was extracted with ethyl acetate (2 x 50 mL) with a small amount of MeOH, dried over Na₂SO₄ and concentrated in vacuum. The solid residue was recrystallized from CH₂Cl₂/*n*-hexane. Yield: 30 mg (0.068 mmol, 43%); colorless solid of m.p. 239–240 °C; ν_{\max} (ATR)/cm⁻¹ 3229, 2924, 2854, 1665, 1621, 1600, 1574, 1521, 1487, 1427, 1394, 1366, 1332, 1266, 1239, 1137, 1105, 1057, 984, 958, 926, 878, 841, 800, 772, 694, 679; ¹H NMR (300 MHz, DMSO-d₆) δ 1.3–1.4 (4 H, m), 1.5–1.6 (2 H, m), 1.6–1.7 (2 H, m), 1.9–2.0 (2 H, m), 7.1–7.2 (1 H, m), 7.3–7.4 (1 H, m), 7.7–8.1 (4 H, m), 8.56 (1 H, s), 8.8–8.9 (1 H, m), 10.6–10.7 (1 H, m); ¹³C NMR (75.5 MHz, DMSO-d₆) δ 24.5, 24.9, 28.4, 32.1, 36.1, 111.8, 115.5, 120.3, 121.3, 122.7, 127.0, 128.1, 129.9, 132.6, 137.3, 141.3, 146.3, 152.8, 157.3, 171.6; m/z (ESI, %) 443.9 (35) [M⁺], 441.9 (100) [M⁺], 428.9 (22), 426.9 (45), 282.1 (35); Anal. calcd. (C₂₂H₂₄ClN₅O₃) C, 59.79; H, 5.47; N, 15.85; Found C, 59.67; H, 5.53; N, 15.69.

Ethyl-8-[4-(3-chloro-4-fluorophenylamino)quinazolin-6-ylamino]-8-oxooctanoate (2c, ClFQuin-SAHEt)

4-(3-Chloro-4-fluorophenylamino)-6-aminoquinazoline (73 mg, 0.254 mmol) was dissolved in dry CH₂Cl₂ and ethyl hydrogen suberate (50 mg, 0.254 mmol). EDCI (75 mg, 0.39 mmol), DMAP (15 mg, 0.10 mmol), and triethyl amine (182 μ L, 0.83 mmol) were added. After stirring at room temperature for 24 h, the solvent was evaporated, and the residue was purified by column chromatography (silica gel 60). Yield: 48 mg (0.102 mmol, 40%); R_f = 0.45 (ethyl acetate); yellow gum; ν_{\max} (ATR)/cm⁻¹ 3335, 3144, 2935, 2861, 1733, 1680, 1617, 1572, 1549, 1498, 1426, 1386, 1365, 1332, 1300, 1242, 1215, 1182, 1129, 1094, 1070, 1058, 1033, 957, 932, 874, 830, 806, 779, 693, 657, 605; ¹H NMR (300 MHz, CDCl₃) δ 1.2–1.4 (7 H, m), 1.5–1.6 (2 H, m), 1.7–1.8 (2 H, m), 2.26 (2 H, t, J = 7.3 Hz), 2.39 (2 H, t, J = 7.3 Hz), 4.11 (2 H, q, J = 7.1 Hz), 7.0–7.1 (1 H, m), 7.3–7.5 (2 H, m), 7.7–7.9 (2 H, m), 8.27 (1 H, m), 8.42 (1 H, m), 8.64 (1 H, s), 8.85 (1 H, s); ¹³C NMR (75.5 MHz, DMSO-d₆) δ 14.1, 24.5, 25.2, 28.6, 34.1, 37.3, 60.5, 110.7, 115.4, 116.2–116.5 (m), 120.7–120.9 (m), 121.7, 124.2, 126.1, 129.2, 135.0, 147.0, 153.1–156.4 (m), 153.9, 157.4, 172.3, 174.2; m/z (%) 474 (43) [M⁺], 472 (95) [M⁺], 427 (23), 288 (100).

N-[4-(3-Chloro-4-fluorophenylamino)quinazolin-6-yl]-N-hydroxyoctanediamide (3c, ClFQuin-SAHA)

Ethyl-8-[4-(3-chloro-4-fluorophenylamino)quinazolin-6-ylamino]-8-oxooctanoate (48 mg, 0.102 mmol) was dissolved in CH₂Cl₂/MeOH (9 mL, 1:2). Hydroxylamine (50% in water, 0.5 mL, 15 mmol) and NaOH (200 mg, 5 mmol) were added, and the reaction mixture was stirred at room temperature for 1 h. The solvent was removed, the residue was dissolved in water, and adjusted to pH 8 with acetic acid. The aqueous phase was extracted with ethyl acetate, dried over Na₂SO₄, and concentrated in vacuum. The solid residue was recrystallized from CH₂Cl₂/*n*-hexane. Yield: 30 mg (0.065 mmol, 64%); colorless solid of m.p. >230 °C (dec.); ν_{\max} (ATR)/cm⁻¹ 3224, 2924, 2852, 1664, 1626, 1572, 1503, 1412, 1333, 1240, 1213, 1138, 1088, 1043, 987, 960, 924, 877, 841, 812, 776, 647, 619; ¹H NMR (300 MHz, DMSO-d₆) δ 1.2–1.3 (4 H, m), 1.4–1.5 (2 H, m), 1.9–2.0 (2 H, m), 2.3–2.4 (2 H, m), 7.3–7.5 (1 H, m), 7.71 (1 H, d, J = 9.0 Hz), 8.0–8.1 (1 H, m), 8.2–8.4 (2 H, m), 8.54 (1 H, s), 9.1–9.2 (1 H, nr s), 11.1–11.2 (1 H, br s); ¹³C NMR (75.5 MHz, DMSO-d₆) δ 24.9, 25.0, 28.3, 32.1, 36.1, 111.9, 115.6, 116.2–116.5 (m), 118.5–118.7 (m), 121.9, 123.0, 126.2, 127.8, 137.9, 145.8, 151.3, 162.6, 157.3, 171.6, 175.0; m/z (ESI, %) 461.9 (40) [M⁺], 459.9 (95) [M⁺], 316.2 (45), 288.2 (70); Anal. calcd. (C₂₂H₂₃ClFN₅O₃) C, 57.46; H, 5.04; N, 15.23; Found C, 57.38; H, 4.98; N, 15.17.

4.3. Biological Evaluations

4.3.1. Cell Culture

Human hepatoma Hep-G2 cells (ATCC number: HB-8065) cells and human prostate cancer DU145 cells (ATCC number: HTB-81) were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (all from Gibco), and grown in an incubator (37 °C, 5% CO₂, humidified atmosphere). Jurkat and SupT11 cell lines were purchased from DSMZ (Braunschweig, Germany). Hut78 cells were purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany), and SMZ-1 cell line was provided by Dr. Raphael Koch from the University Göttingen. The four T-cell leukemia/lymphoma cell lines were cultured in RPMI-1640 medium including L-glutamine (Gibco) supplemented with 10% or 20% fetal bovine serum (Sigma-Aldrich, Darmstadt, Germany) and penicillin/streptomycin at 37 °C in a 5% CO₂ incubator with 95% humidity.

4.3.2. Compounds

Gefitinib and SAHA were purchased from Sigma-Aldrich, (Darmstadt Germany). Stock solutions (10 mM) were prepared in DMSO and stored at −20 °C. Working solutions were always freshly prepared before each experiment by dilution of stock solution with fresh cell culture medium. The final DMSO concentration in each experiment was below 0.25%.

4.3.3. Determination of Growth Inhibition

Treatment-induced changes in cell number were determined by crystal violet (N-hexamethylparosaniline, Sigma Aldrich) staining as described before [35]. In brief, 1000 cells/well (DU145) or 1500 cells/well (Hep-G2) were seeded in 96-well plates and maintained for adherence in an incubator (37 °C, 5% CO₂, humidified atmosphere) for 48 h prior to the beginning of the treatment. Subsequently, cells were treated with rising concentrations (0–10 μM) of the chimeric compounds, SAHA or gefitinib, respectively, for up to 72 h.

Combinatorial drug treatment: HepG2 cells were treated with SAHA and Gefitinib for final concentrations of 0, 0.5, 1, 2.5, 5, and 10 μM for 48 h. Jurkat cells were treated with SAHA for final concentrations of 0, 1.5, 3, and 6 μM and 0, 5, 10, and 20 μM of Gefitinib for 48 h.

Thereafter, the cells were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet. The unbound dye was removed by rinsing with water. Bound crystal violet was solubilized with 0.2% Triton X-100 (Sigma-Aldrich). Light extinction of crystal violet, which increases linearly with the cell number, was analyzed at 570 nm using an ELISA-Reader (Dynex Technologies, Denkendorf, Germany). For T-cell leukemia/lymphoma cell lines IC₅₀ values were determined using dual staining for Annexin-V and 7AAD via flow cytometry [10]. Time- and dose-dependent growth inhibition as well as IC₅₀ values are given as means ± SEM of $n \geq 3$ independent experiments performed in triplicates or more using Graph Pad Prism 8. Synergistic analysis was performed using SynergyFinder 2.0, utilizing the Zero Interaction Potency (ZIP) model [36]. A ZIP score less than -10 means the interaction between two drugs is likely to be antagonistic; from -10 to 10, the interaction between two drugs is likely to be additive; larger than 10, the interaction between two drugs is likely to be synergistic.

4.3.4. Determination of Unspecific Cytotoxicity

To exclude unspecific cytotoxicity as the driving mode of action for antiproliferative effects, the release of lactate dehydrogenase (LDH) from DU145 and Hep-G2 cells was determined after 3 h and 24 h of treatment using the Cytotoxicity Detection Kit PLUS LDH (Roche Diagnostics GmbH, Mannheim, Germany). The assay was performed as described earlier [37]. Adherent non-treated cells, lysed with 2% Triton X-100 media for 10 min, served as reference values for maximum LDH release. Cytotoxicity was determined by subtracting the percentage of LDH release into the supernatant under control conditions of those

from treated samples. Measurements were performed in duplicate in $n = 3$ independent experiments and mean percentage changes \pm SEM as compared to controls are shown.

4.3.5. EGFR Kinase Inhibition

To determine the EGFR tyrosine kinase-inhibitory potency of 3BrQuin-SAHA and 3ClQuin-SAHA, a cell-free EGFR kinase assay from Promega was employed (#3831) [38]. The clinically approved EGFR tyrosine kinase-inhibitor gefitinib (1 μ M) was used as a positive control. Kinase reactions were carried out with EGFR (5 ng/ μ L), ATP (5 μ M), and substrates (0.2 μ g/ μ L) in kinase reaction buffer using kinase enzyme systems (Promega, Madison, WI, USA). Before the kinase reaction was started, enzyme and inhibitors were incubated at room temperature (22–25 $^{\circ}$ C) for 0.5 h. All kinase reactions were performed in 384-well plates with a volume of 5 μ L and an incubation time of 1 h. To deplete the remaining ATP, 5 μ L of ADP-Glo reagent (ADP-Glo kinase assay kit; Promega) was added to each well at RT for 40 min. Finally, 10 μ L of kinase detection solution was added into each well of the 384-well plate. Luminescence was measured with a VarioSkan Flash 40053 microplate luminometer (Thermo Fisher Scientific, Waltham, Mass., USA) for 1 s. Measurements were performed in triplicate. Data of $n = 3$ independent experiments are given as means \pm SEM of the percentage decrease in EGFR-TK activity, as compared to untreated controls, whose activity was set to 100%.

4.3.6. Inhibition of HDAC Activity

The HDAC-inhibitory potential of 3ClQuin-SAHA and 3BrQuin-SAHA was determined by using fluorogenic HDAC assay kits for the detection of pan-HDAC activity (Calbiochem, Merck Chemicals, Darmstadt, Germany) or the subtype-specific HDAC 2 and HDAC 6 activity (BPS Biosciences, San Diego, CA, USA). HDAC activity was measured according to the instructions of the supplier. SAHA (1–10 μ M) served as positive control. Human HDAC enzymes derived from HeLa cell nuclear extracts (pan HDAC assay) or human recombinant HDAC-2 and HDAC-6 enzymes and adjacent fluorogenic HDAC substrates were used to determine HDAC activity levels. A 50 μ L assay buffer containing 1 μ g/ μ L bovine serum albumin, the human HDAC enzymes, 3ClQuin-SAHA (1–10 μ M), 3BrQuin-SAHA (1–10 μ M) or gefitinib (1–10 μ M), and the corresponding HDAC substrates were added into a black 96-well assay plate. The reaction in each well was incubated at 37 $^{\circ}$ C for 30 min, followed by adding 50 μ L HDAC developer reagent, and incubated at room temperature for an additional 15 min. Fluorescence intensity of the assay plates was measured on a Varioskan Flash fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

4.3.7. Measurement of Apoptosis-Specific Caspase-3 Activity

Changes in caspase-3 activity were measured by the cleavage of the fluorogenic substrate AC-DEVD-AMC (EMD Millipore, Billerica, MA, USA), as described previously [37]. After incubation with 3BrQuin-SAHA, 3ClQuin-SAHA, SAHA, or gefitinib (1–10 μ M) for 6–48 h, the cells were harvested and lysed with lysis buffer. Subsequently, the lysates were incubated for 1 h at 37 $^{\circ}$ C with a substrate solution containing 20 μ g/mL AC-DEVD-AMC, 20 mM HEPES, 10% glycerol, and 2 mM DTT at pH 7.5. Substrate cleavage was measured fluorometrically using a Varioskan Flash fluorometer (Thermo Fisher Scientific, Waltham, MA, USA; filter sets: ex 360/40 nm, em 460/10 nm). Three independent measurements were performed in triplicate, and data are given as the mean percentage increase \pm SEM above control, which was set to 100%.

4.3.8. Measurement of Reactive Oxygen Species (ROS)

The formation of cytosolic ROS in DU145 and Hep-G2 cells after treatment with 3ClQuin-SAHA, 3BrQuin-SAHA, SAHA or gefitinib (10 μ M) was measured using the membrane permeable dye CellROX[®] Orange (Thermo Fisher Scientific) which accumulates in the cytoplasm, exhibiting strong fluorescent signals at excitation/emission levels of

545 nm/565 nm upon oxidation [39]. Untreated cells incubated with 1.6 mM H₂O₂ for 30 min served as positive controls. CellROX[®] Orange reagent (1 μM) was applied, when adding the test compounds. Formation of ROS was measured after 24 h using ZOE[™] Fluorescent Cell Imager (Biorad, Munich, Germany). Independent experiments were performed in triplicate for each condition.

4.3.9. Western Blot

Briefly, Western blots were performed as described earlier [40]. PCa cells were seeded in 100 mm petri dishes, grown to almost confluency, treated for 24 h, washed and frozen, followed by lysis with RIPA Buffer, added with one cOmplete[™] Mini Protease Inhibitor tablet/10 mL (Roche), and quantification with Pierce[™] BCA Protein Assay Kit (Thermo Fisher Scientific). Thereafter, protein levels were normalized to untreated controls, so protein-loading was equal to 20 μg/lane. Laemmli Buffer and β-mercaptoethanol were added before probes were desaturated at 96 °C for 10 min. First, 7.5% or 12% SDS gels (Biorad, Munich, Germany) were loaded with proteins and electrophoresis was done. Thereafter, proteins were transferred to activated polyvinylidene difluoride membranes (PVDF) by electroblotting. Finally, the membranes were blocked with 1% BSA and incubated with primary antibodies over night at 4 °C. The following antibodies directed against HDAC2 (5113S Cell signaling, 1:1000), HDAC6 (7558S Cell signaling, 1:1000), acetylated histone H3 (ab47915 abcam, 1:1000), EGFR (sc03 Santa Cruz Biotechnology, 1:500), poly-(ADP-ribose)-polymerase (PARP), and cleaved PARP (11835238 Roche, 1:1000), and β-actin (A5441 Sigma Aldrich, 1:2000) for standardization were used. Blots were washed three times with 1% TBS-Tween and incubated with anti-mouse or anti-rabbit peroxidase-coupled anti-IgG secondary antibodies (1:500–1:1000) at room temperature for 60 min. Subsequently, antibody bondage was illustrated using Clarity and Clarity Max ECL Western Blotting Substrates (Biorad, Munich, Germany) for detection and Celvin-S developer (Biostep, software SnapAndGo) for development. Independent blots of $n = 3$ experiments were generated, and the expression levels were determined by analyzing the bands with ImageJ and calculating the area under the curve (AUC) relative to its greyscale. Values were normalized to beta-actin expression, which served as a loading control, and compared to untreated control expressions, which was set 1. The mean ± SEM of the relative values to control are illustrated with Graph Pad Prism 8.

4.3.10. In Vivo/Ovo Evaluation of Angiogenesis

Effects of 3BrQuin-SAHA and 3ClQuin-SAHA on angiogenesis were investigated on the CAM (chorioallantoic membrane) of fertilized chicken eggs [41]. Embryonic development is initiated by putting the eggs in an upright position into a humidified (>60%) incubator at 37.8 °C. At day 3 of egg development, a small 2 mm hole is pierced in the top of the eggshell, causing the developing CAM to detach from the eggshell and lower itself into the allantoic cavity. At day 10, the eggshell is further opened, and a silicone ring (1 cm Ø) is placed carefully on a region of interest of the CAM and allowed to attach for 6 h. Thereafter, 20 μL of inhibitor containing medium or PBS (control) is pipetted carefully into the ring. Changes in the angiogenic state of the CAM is then documented microscopically using a stereomicroscope equipped with a Kappa digital camera system (Distelkamp-Electronic, Kaiserslautern, Germany) by taking pictures every 24 h until the end of the experiment after 3 days of incubation. Experiments were performed with $n = 3$ eggs for each condition.

4.3.11. Statistical Analysis

Statistical calculations were done with GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) using two-way ANOVA Dunnett's post hoc test for statistical significance testing or linear regression.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/ijms22168432/s1>, Figure S1: EGFR expression in T-cell lymphoma cell lines.

Author Contributions: Conceptualization: M.H. (Michael Höpfner), B.N., and B.B.; methodology, N.G., Y.P., M.H. (Marco Herling), and A.M.; validation, M.H. (Michael Höpfner), B.N., B.B., and R.S.; investigation, N.G., Y.P., M.H. (Marco Herling), and A.M.; data curation, M.H. (Michael Höpfner), B.N., and B.B.; writing—original draft preparation, N.G., B.N., M.H. (Michael Höpfner); writing—review and editing, M.H. (Michael Höpfner), R.S., N.G., and B.N.; visualization, N.G., B.N., B.B., and M.H. (Michael Höpfner); supervision, M.H. (Michael Höpfner); project administration, M.H. (Michael Höpfner). All authors have read and agreed to the published version of the manuscript.

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Abbreviations

CAM	Chorioallantoic membrane
EGFR	Epidermal growth factor receptor
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
LDH	Lactate dehydrogenase
PCa	Prostate cancer
ROS	Reactive oxygen species

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Article

Improved Anticancer Activities of a New Pentafluorothio-Substituted Vorinostat-Type Histone Deacetylase Inhibitor

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Abstract: The development of new anticancer drugs is necessary in order deal with the disease and with the drawbacks of currently applied drugs. Epigenetic dysregulations are a central hallmark of cancerogenesis and histone deacetylases (HDACs) emerged as promising anticancer targets. HDAC inhibitors are promising epigenetic anticancer drugs and new HDAC inhibitors are sought for in order to obtain potent drug candidates. The new HDAC inhibitor SF5-SAHA was synthesized and analyzed for its anticancer properties. The new compound SF5-SAHA showed strong inhibition of tumor cell growth with IC₅₀ values similar to or lower than that of the clinically applied reference compound vorinostat/SAHA (suberoylanilide hydroxamic acid). Target specific HDAC inhibition was demonstrated by Western blot analyses. Unspecific cytotoxic effects were not observed in LDH-release measurements. Pro-apoptotic formation of reactive oxygen species (ROS) and caspase-3 activity induction in prostate carcinoma and hepatocellular carcinoma cell lines DU145 and HepG2 seem to be further aspects of the mode of action. Antiangiogenic activity of SF5-SAHA was observed on chorioallantoic membranes of fertilized chicken eggs (CAM assay). The presence of the pentafluorothio-substituent of SF5-SAHA increased the antiproliferative effects in both solid tumor and leukemia/lymphoma cell models when compared with its parent compound vorinostat. Based on this preliminary study, SF5-SAHA has the prerequisites to be further developed as a new HDAC inhibitory anticancer drug candidate.

Keywords: fluorine; histone deacetylase inhibitor; anticancer drugs

1. Introduction

Histone deacetylases (HDACs) are epigenetic regulators of chromatin (de-)condensation and, thus, play an important role in various crucial cellular processes [1]. HDACs are over-expressed in various cancers and exert a strong impact on cancer cell proliferation, dissemination and metastasis [2]. Hence, HDAC inhibitors are a promising class of compounds for targeted cancer therapy. Some HDAC inhibitors such as vorinostat/SAHA (suberoylanilide hydroxamic acid) and panobinostat are already approved for the treatment of hematologic cancer and are currently under intensive investigation for their suitability in solid tumors [3]. However, severe drawbacks have emerged during the clinical application of single HDAC inhibitors such as intrinsic or acquired drug resistance. Hence, the search for new HDAC inhibitors with improved activities has become a relevant and prospering field of anticancer

research and several HDAC inhibitors with promising effects on prostate cancer and liver cancer were recently described [4–8].

Fluorine has a prominent role for the fine-tuning of drugs and fluorine substituents significantly altered activity, conformation, pK_a , membrane permeability, and pharmacokinetics of drug candidates [9]. Aside established ^{19}F -PET methods, fluorination was also successfully applied for bioanalytical labelling in drug uptake studies using molecular absorption spectrometry [10]. The pentafluorothio group is a lipophilic and electron-withdrawing substituent, which is a xenobiotic, chemically stable mimic of negatively charged biomolecules [11]. The 8-pentafluorothio analog of the antimalarial drug mefloquine is a prominent example with a higher antimalarial in vivo activity and a longer half-life than mefloquine [11,12]. Recently, our group has disclosed the positive effect of SF_5 substituents on the antitumoral activity of curcuminoids [13,14]. The SAHA molecule was modified at the *para*-position with various halogen substituents of the cap phenyl ring without loss of activity when compared with SAHA [15]. However, a *para*- SF_5 -aryl capped vorinostat derivative was not reported until now.

In the present report, a new analog of SAHA with a 4-pentafluorostulfanyl substituted cap phenyl ring is prepared, and its anticancer activities and modes of action are described in comparison with the parent compound SAHA as a reference compound (Figure 1).

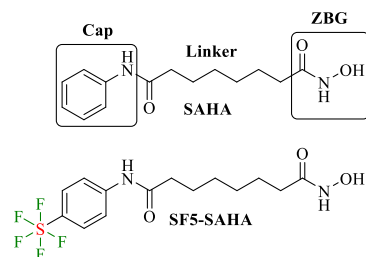
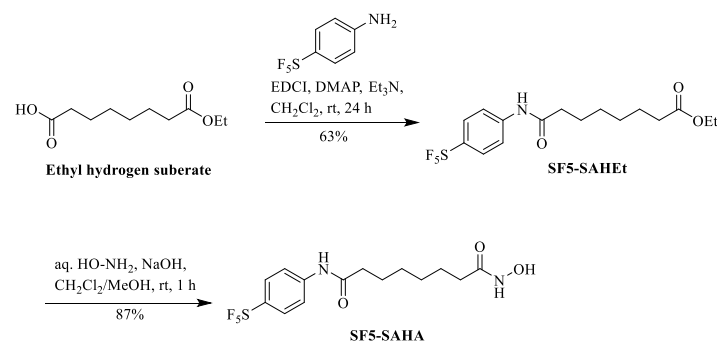


Figure 1. Structures of SAHA (vorinostat) and the new analog SF5-SAHA (ZBG = zinc-binding group).

2. Results

2.1. Chemistry

Ethyl hydrogen suberate was reacted with 4-pentafluorothioaniline and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) to afford the ethyl ester precursor SF5-SAHet in moderate yield (63%) (Scheme 1). Target compound SF5-SAHA was obtained in high yield (87%) from SF5-SAHet in aqueous hydroxylamine under basic conditions.



Scheme 1. Synthesis of SF5-SAHA.

2.2. Biological Evaluation

2.2.1. Antiproliferative Activity

Initially, the new compound SF5-SAHA was tested for its growth inhibitory effects on human DU145 prostate carcinoma cells (androgen-independent prostate cancer cells) and human Hep-G2 hepatoblastoma cells representing solid cancer entities with a high need for expanded medical treatment options (Table 1). The results were compared with data of the parent compound SAHA as a reference compound [16]. The DU145 cells were sensitive to both test compounds and SF5-SAHA showed virtually the same activity as SAHA in these cells. In contrast to that, SF5-SAHA was almost twice as active as SAHA against Hep-G2 cells.

Table 1. Growth inhibitory activity expressed as IC₅₀ (μM) of test compound SF5-SAHA and of SAHA as positive control in human prostate (DU145) and hepatoma (Hep-G2) cancer cells as well as in human T-cell leukemia/lymphoma cell lines (Jurkat, Hut78, SupT11, SMZ1), as determined after 48 h. SAHA was used as clinically relevant reference for single HDAC-inhibition. All results are described as means ± SEM of n ≥ 3 independent experiments.

Compounds	DU145	Hep-G2	Jurkat	Hut78	SupT11	SMZ1
SF5-SAHA	0.73 ± 1.08	1.79 ± 1.06	0.76 ± 0.06	2.40 ± 0.40	3.08 ± 0.33	1.58 ± 0.29
SAHA	0.68 ± 0.04	3.22 ± 0.44	1.70 ± 0.17	5.07 ± 0.42	4.67 ± 0.31	2.87 ± 0.33

Lymphomas and leukemias are hematological malignancies where single HDAC inhibitors such as SAHA or panobinostat are either already approved (T-cell leukemias/lymphomas) or show eminent pre-clinical data [17]. Hence, SF5-SAHA was also tested for its activity against a panel of lymphoma and leukemia cell lines (Jurkat, Hut78, SupT11 and SMZ1) and the results were compared with corresponding data of SAHA again. The activity (IC₅₀ values) of SF5-SAHA was in the sub-μM concentration range for the Jurkat cells and in the low single-digit μM concentration range for the other three cell lines Hut78, SupT11, and SMZ1 (Table 1). Interestingly, SF5-SAHA was more active than SAHA, which served as a reference for a clinically approved HDAC-inhibitor, against cells of these four cell lines. Hence, the novel inhibitor SF5-SAHA was shown to have superior antiproliferative potency in hematologic tumor cell models, too.

2.2.2. Unspecific Cytotoxicity of SF5-SAHA

To check for unspecific cytotoxic effects possibly contributing to the antiproliferative effects of SF5-SAHA treatment, the release of lactate dehydrogenase (LDH) from the cytosol into the supernatant of DU145 or Hep-G2 cell cultures was measured. Increased LDH release indicates unspecific and necrotic cell death due to a treatment-induced damage of cell membranes [18]. However, SF5-SAHA did not induce significant increases in LDH release after 3 and 24 h of treatment with rising compound concentrations (1–10 μM) (Figure 2). In Hep-G2 cells, LDH levels were even decreased upon treatment with SF5-SAHA. The data indicate that even at high concentrations SF5-SAHA does not affect cell membrane integrity. Thus, an induction of immediate and unspecific cytotoxicity is unlikely to account for the observed antiproliferative effects of the compound. Moreover, as expected for the reference compound SAHA, no relevant induction of unspecific cytotoxicity was observed.

2.2.3. Apoptosis Induction by SF5-SAHA

To evaluate if apoptosis may play a role in the antiproliferative effects of SF5-SAHA, the activation of the apoptosis specific effector caspase-3 in Hep-G2 and DU145 cells was investigated. Upon SF5-SAHA treatment, a pronounced caspase-3 activation became apparent. After 24 h of treatment, an upregulation of up to 5–6 times of the activity of untreated cells was observed (Figure 3A). Treatment with SAHA evoked comparable caspase-3 inductions. Western Blot analyses revealed a concomitant induction of apoptosis specific cleavage of poly (ADP-ribose)-polymerases (PARP) (Figure 3B,C). PARP cleavage is

caspace 3-driven and may be used as a marker for chemotherapy-induced apoptosis [19,20]. In Hep-G2 cells, PARP cleavage experiments led to inconclusive results. PARP expression was suppressed, but no cleaved PARP was detected in Hep-G2 cells treated with SF5-SAHA. Thus, we studied the expression levels of further important apoptosis-related proteins such as pro-apoptotic Apaf-1 and anti-apoptotic Bcl-2. SF5-SAHA treatment increased Apaf-1 expression at doses of 2 and 4 μM while anti-apoptotic Bcl-2 expression was downregulated by 2 μM and 4 μM SF5-SAHA (Figure 3D). Our findings affirmed the hypothesis that apoptosis may play a prominent role for the effects that we observed upon treatment of Hep-G2 and DU145 cells with SF5-SAHA.

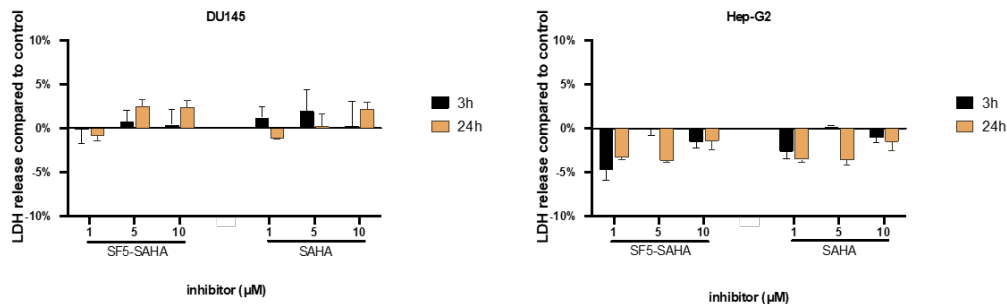


Figure 2. Unspecific cytotoxic effects of SF5-SAHA in DU145 prostate cancer and Hep-G2 hepatoblastoma cells. LDH release of cells was measured after 3 and 24 h of incubation with 1, 5 or 10 μM of SF5-SAHA or SAHA. Results show changes in LDH release relative to untreated controls. Data are given as percentage changes relative to basal LDH release of controls. Means \pm SEM of $n = 3$ independent experiments.

2.2.4. ROS Induction by SF5-SAHA

For a deeper insight into the molecular events underlying the mode of action of SF5-SAHA, we investigated a possible involvement in the induction of the formation of reactive oxygen species (ROS), since HDAC inhibition has already been shown to be linked to ROS induction in solid cancers, including prostate cancer [21]. Treatment of DU145 and Hep-G2 cells with SF5-SAHA led to a pronounced time- and dose-dependent increase in cytosolic ROS after 6–24 h, as evidenced by fluorescence microscopy with the cytosol-specific ROS-dye CellROX orange. Notably, a similar treatment with equimolar concentrations of SAHA elicited a much weaker ROS increase in prostate and hepatocellular cancer cells (Figure 4).

2.2.5. HDAC Inhibition by SF5-SAHA

To assess the HDAC inhibitory potency of SF5-SAHA, commercially available HeLa cell nuclear extracts constituting a cell free pan-HDAC enzyme profile, were treated with SF5-SAHA (1–10 μM). The compound strongly inhibited the HDAC activity in a dose-dependent manner leading to IC_{50} values in the submicromolar range. Compared with SAHA ($\text{IC}_{50} = 20 \text{ nM}$), the mainstay of HDAC-targeting anticancer therapy, the pan-HDAC-inhibitory potency of the novel compound is only slightly weaker (Figure 5A). The HDAC-inhibitory efficacy was further evaluated in prostate and liver cancer cells by immunodetection of the increased portion of acetylated histone H3 which is part of the cellular nucleosome. Western Blot analysis revealed a dose-dependent rise of H3 acetylation of DU145 and Hep-G2 cells upon treatment with SF5-SAHA or SAHA as a consequence of the suppression of the histone deacetylating activity of HDACs (Figure 5B,C).

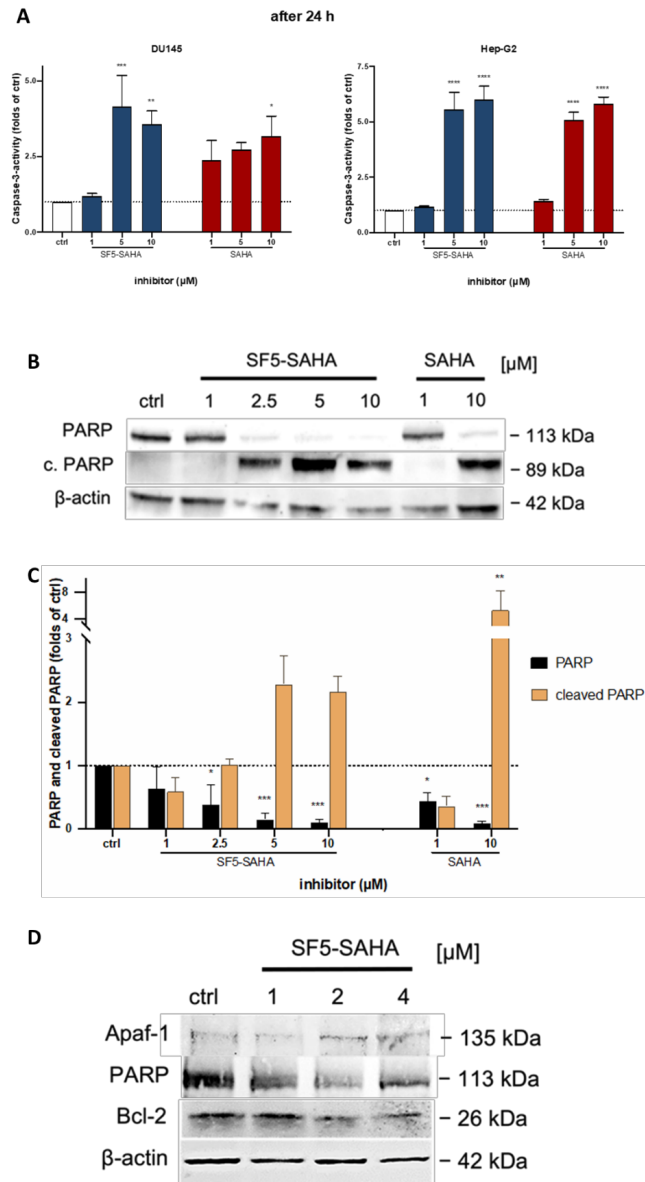


Figure 3. Apoptosis induction by SF5-SAHA. (A) Caspase-3 induction in DU145 or Hep-G2 cells after 24 h treatment with SF5-SAHA and SAHA. Means \pm SEM of $n = 3$ independent experiments. (B) Representative Western blot out of $n = 3$ experiments, showing changes in the expression of PARP and PARP after cleavage (c. PARP) in DU145 cells after 24 h treatment with test compounds. (C) From these Western blots gray intensities, of PARP and cleaved PARP specific bands were quantified adjusted to protein loading and normalized to untreated controls. (D) Representative Western blot out of $n = 3$ experiments, showing changes in the expression of Apaf-1, PARP, and Bcl-2 in Hep-G2 cells after 24 h treatment with SF5-SAHA. Data are given as means \pm SEM of $n = 3$ independent experiments. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$, **** $p \leq 0.0001$; 2-way ANOVA Dunnett's post-hoc test.

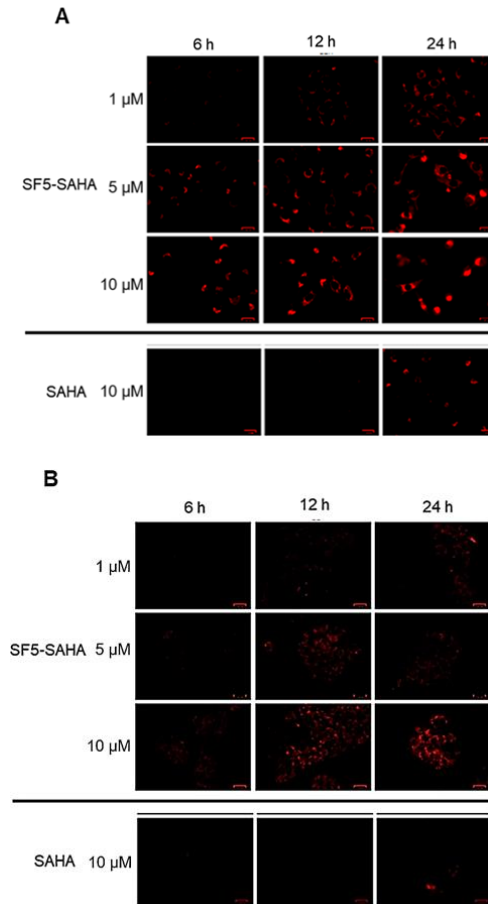


Figure 4. ROS induction (fluorescence microscopy using the ROS-dye CellROX orange) after 6 h, 12 h, and 24 h of incubation with SF5-SAHA and SAHA in DU145 (A) and Hep-G2 cells (B). Orange fluorescence indicates oxidation of the ROS-dye by formed ROS.

The levels of acetylated histone H3 and α -tubulin were also investigated in T-cell leukemia/lymphoma cells upon treatment with SF5-SAHA for 24 h (Figure 6). Dose-dependent increases in acetylated H3 were observed in all tested leukemia/lymphoma cancer cell lines. Except for the Jurkat cells, increased acetyl-H3 levels were already observed at the lowest SF5-SAHA dose of 1 μ M when compared with untreated control cells. Acetyl- α -tubulin levels also increased in a dose-dependent way in three out of four tested cell lines, which was in line with the inhibitory effects of SF5-SAHA on cytoplasmic HDAC6. Only the Jurkat cells already showed a high level of α -tubulin in untreated cells and, thus, drug-induced changes were not detectable in these cells.

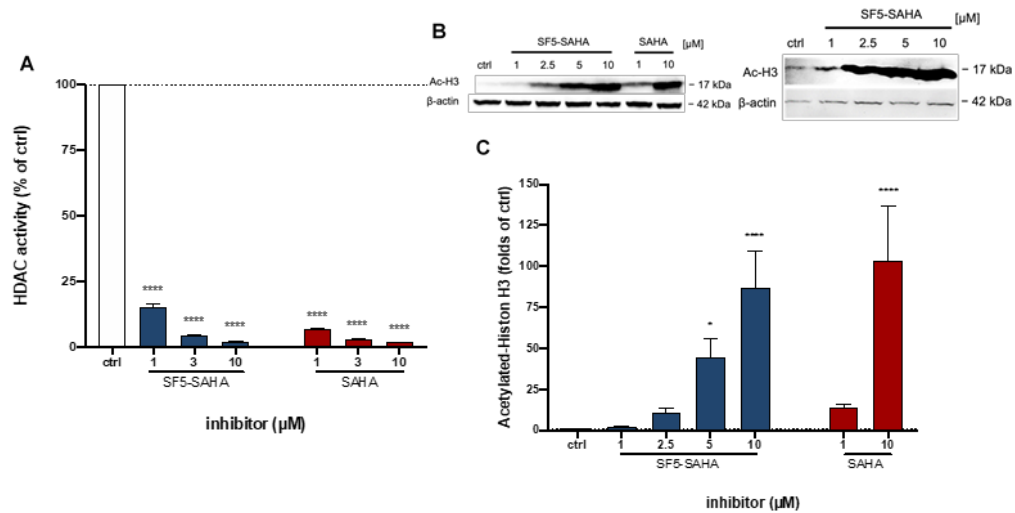


Figure 5. HDAC inhibitory effects of SF5-SAHA in cancer cells. (A) HDAC activity was measured by luminescence display of the acetylation state of histones. HeLa nuclear cell extracts, used as source for histones, were preincubated with substrate and inhibitor. Results are given as relative to controls, not preincubated with inhibitor, as means \pm SEM of $n = 3$ independent experiment. (B) Representative Western blot out of $n = 3$ experiments, showing changes in the expression of acetylated histone H3 after 24 h treatment with compounds in DU145 (left) and Hep-G2 (right) cells. (C) From the Western blots gray intensity, mean \pm SEM, of acetylated histone H3 specific bands were quantified adjusted to protein loading and normalized to untreated control. * $p < 0.05$, **** $p < 0.0001$; 2-way ANOVA Dunnett's post-hoc test.

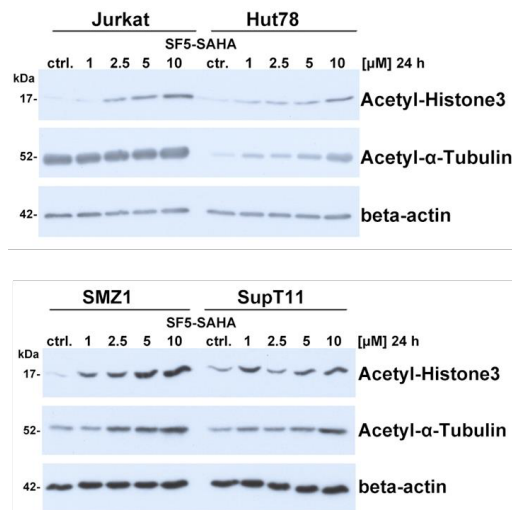


Figure 6. HDAC inhibitory effects of SF5-SAHA in T-cell leukemia/lymphoma cells (Jurkat, Hut78, SMZ1, SupT11). Representative Western blots out of $n = 3$ experiments, showing changes in the expression of acetylated histone H3 and acetylated α -tubulin after 24 h treatment with SF5-SAHA.

The subtype-specific inhibition of HDAC1, HDAC2 and HDAC6 by SF5-SAHA was evaluated using cell-free enzymatic HDAC assays and compared with the activity of SAHA (Figure 7). SF5-SAHA exhibited distinctly stronger HDAC6 inhibition than SAHA. In con-

trast, SAHA performed slightly better than SF5-SAHA in terms of HDAC2 inhibition. Both compounds showed comparable activities against HDAC1. However, the inhibitory effects of both compounds were less pronounced than against HDAC2 and HDAC6. Western blot experiments confirmed that both DU145 cells and Hep-G2 cells express HDAC1, HDAC2, and HDAC6 enzymes as conceivable targets of SF5-SAHA in these cell lines (Figure 8).

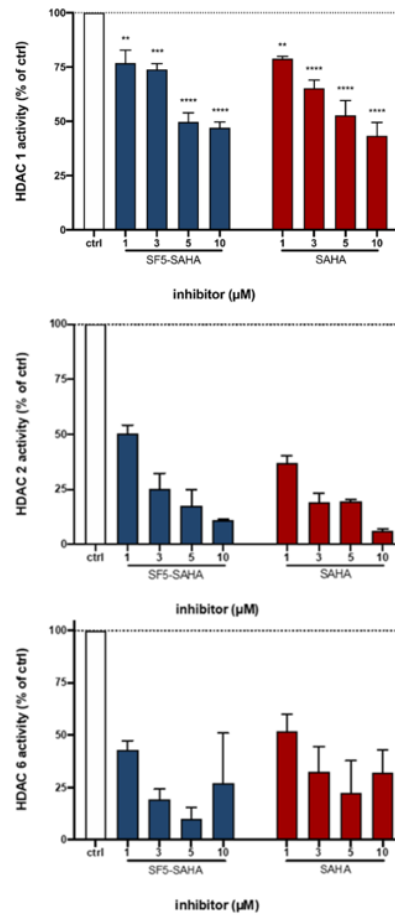


Figure 7. Inhibition of HDAC1 (top), HDAC2 (middle) and HDAC6 (bottom) by SF5-SAHA and SAHA. HDAC activity was measured by luminescence display of the acetylation state of histones. Human recombinant HDAC1, HDAC2 and HDAC6 enzymes and adjacent fluorogenic HDAC substrates were used to determine subtype specific HDAC activity levels after preincubation with SF5-SAHA and SAHA. Results are given as relatives to controls, not preincubated with inhibitor, as means \pm SEM of $n = 3$ independent experiments. ** $p \leq 0.005$, *** $p \leq 0.0005$, **** $p \leq 0.0001$; 2-way ANOVA Dunnett's post-hoc test.

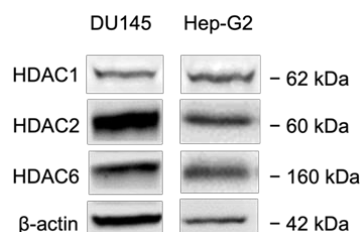


Figure 8. Western blot determined expression of HDAC1, HDAC2, and HDAC6 in DU145 prostate cancer and Hep-G2 liver cancer cells.

Docking studies of SF5-SAHA bound to the active site of HDAC2 (PDB ID 4LXZ) were performed in order to determine the binding mode of SF5-SAHA (Figure 9). The molecular docking was carried out in AutoDock Vina in the presence of the catalytic Zn^{2+} ion at the binding site. As expected, SF5-SAHA binds HDAC2 in a way very similar to SAHA. The binding modes of SAHA and SF5-SAHA are similar because the structural difference of both ligand molecules is based on the solvent exposed moieties and not on the ZBG and linker systems. Both molecules coordinate the Zn^{2+} ion via their hydroxamic acid ZBG. Interestingly, the calculated HDAC2 binding energy of SF5-SAHA was higher (-7.4 kcal/mol) than of SAHA (-6.7 kcal/mol).

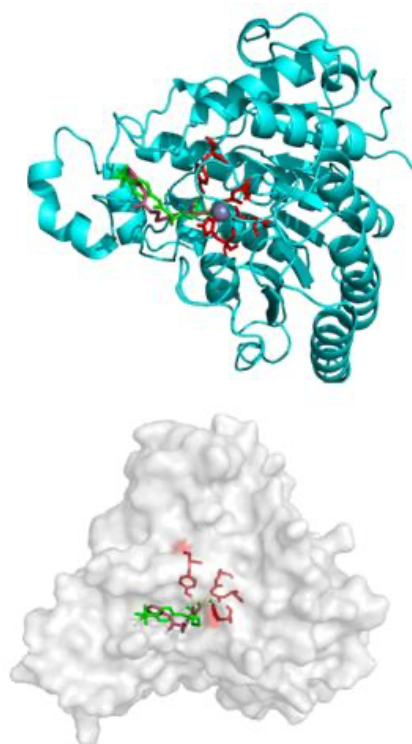


Figure 9. Overlay images of docked SF5-SAHA (green) and SAHA (purple) with HDAC2 in cartoon view (top image) and surface view (bottom image). The interaction of inhibitors with active site amino acids (red) and zinc ion (sphere) is highlighted.

2.2.6. Effects on EGFR Signaling

We checked for cellular effects of SF5-SAHA by determination of changes in the expression level of the EGFR in DU145 prostate cancer cells. Western Blots for the EGFR expression level of treated vs. non-treated DU145 cells revealed that SF5-SAHA led to a significant downregulation of EGFR protein expression (Figure 10A,B). Treatment of DU145 cells with SAHA also led to a pronounced and significant suppression in the expression of the EGFR. Thus, we could show that the hydroxamate pharmacophore contributes to a significant suppression of the expression of the EGFR because the non-modified EGFR inhibitor gefitinib, which does not inhibit HDACs, showed no EGFR-suppressing effects. In addition, SF5-SAHA reduced the level of phosphorylated (activated) mTOR (P-mTOR) similar to SAHA and gefitinib while the mTOR level itself was only slightly reduced by SF5-SAHA and SAHA.

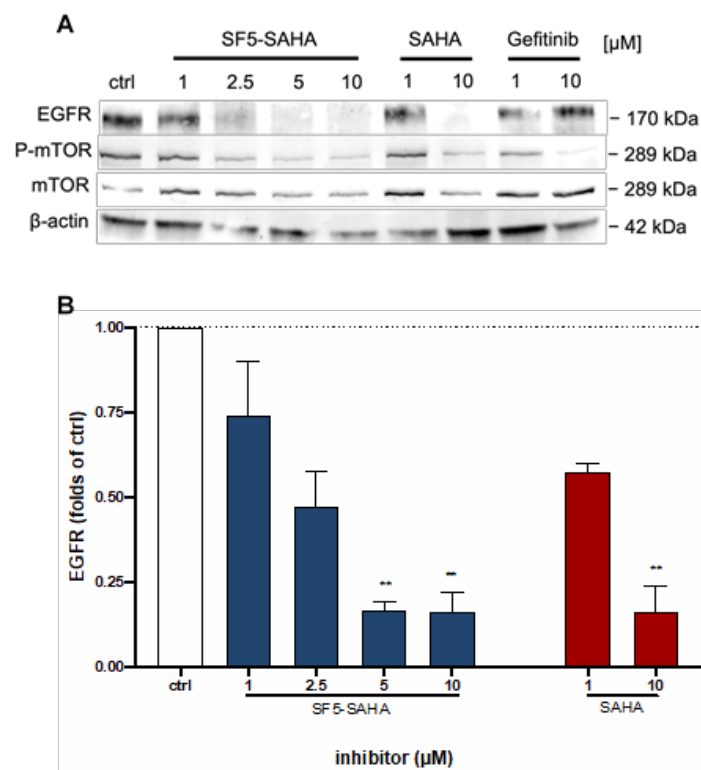


Figure 10. Inhibitory effects of SF5-SAHA on EGFR signaling pathway protein expression. (A) Representative Western blots of $n = 3$ independent experiments showing treatment induced changes in the expression of EGFR in DU145 cells after 24 h. β -actin was used as loading control. (B) From these Western blots gray intensity, mean \pm SEM, of EGFRs specific bands were quantified and adjusted to protein loading and normalized to untreated control. ** $p \leq 0.005$; 2-way ANOVA Dunnett's post-hoc test.

2.2.7. Antimigration Effects

Scratch assays were performed in order to study the anti-migratory activity of SF5-SAHA in DU145 prostate tumor cells (Figure 11). After incubation with SF5-SAHA for 24 h, cells clearly showed a reduced migratory activity at doses of 5 μ M and 10 μ M when compared with untreated cells, where the initial scratch was largely healed.

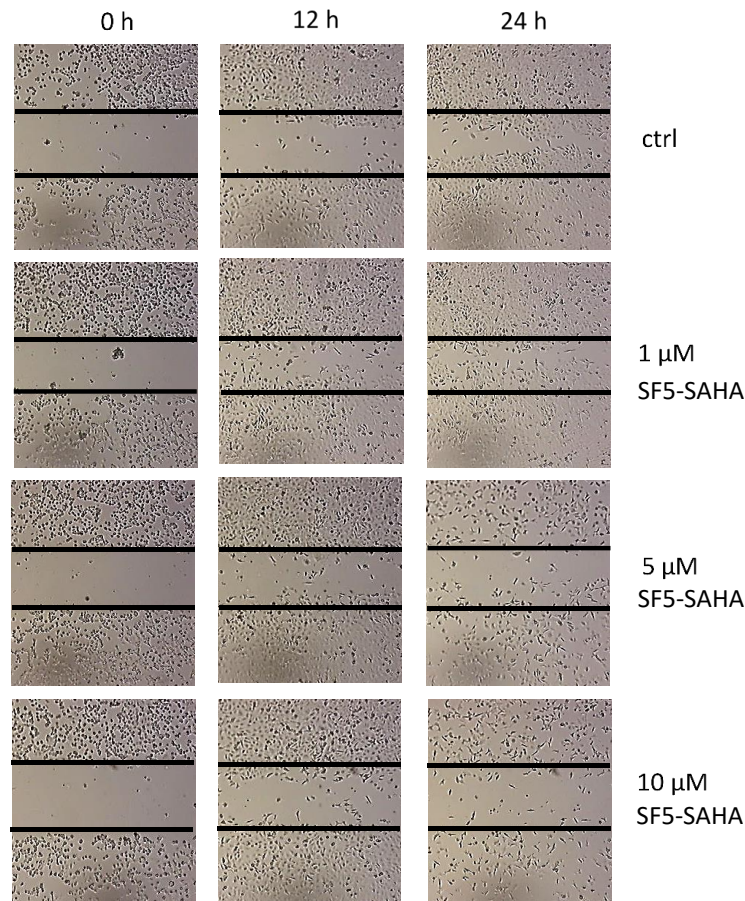


Figure 11. Inhibitory effects of SF5-SAHA on DU145 tumor cell migration. Representative scratch assay images of $n = 3$ independent experiments showing a treatment induced retarded migration of DU145 cells after 24 h. Black lines indicate the initial scratch areas at the corresponding starting points (0 h). Control cells (ctrl) are untreated cells in medium.

2.2.8. Antiangiogenic Effects

Angiogenesis experiments using the chorioallantoic membrane (CAM) of fertilized chicken eggs were carried out in order to study the antiangiogenic effects of SF5-SAHA (Figure 12). After topical application of SF5-SAHA and incubation for 72 h, the CAM clearly showed reduced vessel diameters and reduced numbers of vessel branches when compared with untreated eggs. Hence, SF5-SAHA also conserved the previously reported antiangiogenic effects of SAHA in the CAM assay [16]. In addition, no toxicity of SF5-SAHA to chicken embryos was observed at the indicated doses of 5 and 10 μM .

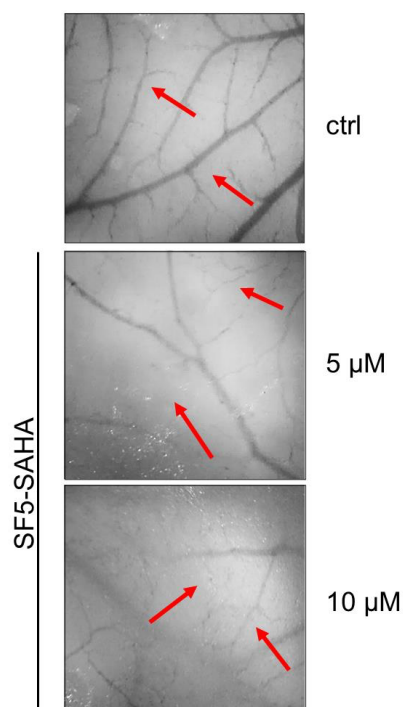


Figure 12. Angiogenesis in fertilized chicken eggs (CAM assay). Representative images of $n = 4$ independent experiments showing antiangiogenic effects of SF5-SAHA (5 or 10 μM) after 72 h. Control (ctrl) image shows vessels of untreated CAM (PBS control). Red arrows indicate conspicuous branches and vessel diameters.

3. Discussion

SAHA (vorinostat) is a clinically applied HDAC inhibitor. In this study, we have shown that the novel pentafluorothio-substituted SF5-SAHA is more active than SAHA against cancer cells. SAHA performed best in the DU145 prostate carcinoma cells and SF5-SAHA displayed a very similar IC_{50} value in antiproliferative activity assays with these cells. SF5-SAHA was distinctly more active than SAHA against all other cell lines tested in this study. Hence, SF5-SAHA appears to be a suitable alternative to or surrogate of SAHA for the treatment of cancers, which are less susceptible to SAHA treatment. Apoptosis induction is a cell death mode of action of many anticancer agents and a hallmark of drug sensitive cancers [22]. In the sensitive DU145 cells, SF5-SAHA induced apoptosis via caspase-3 activation. After 24 h, SF5-SAHA strongly induced apoptosis both in DU145 and in Hep-G2 cells, albeit slightly less than SAHA. The pro-apoptotic effect of SF5-SAHA was confirmed by detection of PARP cleavage in treated DU145 cells. In addition, Bcl-2 was suppressed and Apaf-1 was upregulated in Hep-G2 cells treated with SF5-SAHA. Apaf-1 is a crucial factor of the apoptosome and the associated mitochondrial-caspase apoptosis induction pathway. Previous reports showed that the natural HDAC inhibitor trichostatin A (TSA) upregulated Apaf-1 accompanied by apoptosome activation in HCC cells [23]. Hence, SF5-SAHA conserved the apoptosis inducing properties of HDAC inhibitors such as SAHA and TSA. Apoptosis induction is a crucial mechanism of the anticancer effect of SAHA and acquired SAHA-resistance of colon cancer cells was shown to be a consequence of the loss of their apoptosis propensity [24]. While SF5-SAHA induced apoptosis, it showed no unspecific toxicity in the tested cancer cells. However, SF5-SAHA owns further

modes of action. For instance, SF5-SAHA led to much higher ROS levels in treated cells than SAHA, which might explain its increased antiproliferative effect in the Hep-G2 cells when compared with that of SAHA. In contrast to that, the increased ROS formation upon treatment with SF5-SAHA probably does not play a distinguishing role for the DU145 cells. HDAC inhibition has already been shown to be linked to ROS induction in solid cancers, including prostate cancer [21]. In addition, the redox modulator β -phenylethyl isothiocyanate resensitized SAHA-resistant leukemia cells by inhibition of glutathione and suppression of the cellular antioxidant-based resistance mechanism [25]. ROS induction has also been linked to the activation of caspases and may serve as a trigger of apoptosis [26]. Hence, the higher ROS formation induced by SF5-SAHA when compared with the ROS formation elicited by SAHA is of great significance.

SF5-SAHA is a strong inhibitor of HDACs with an activity similar to that of SAHA in DU145 cells. HDAC enzymes are important targets of anticancer therapy. The increased expression of HDACs in prostate cancer and HCC was correlated with lower survival rates [27,28]. HDAC2 upregulation, for example, was a predictor for HCC patient survival [29]. HDAC2 expression was found crucial for anti-apoptotic effects and HDAC2 inhibition increased apoptosis in colon carcinoma cells [30,31]. HDAC inhibition by SAHA also led to an enhanced apoptosis induction in prostate tumor cells [32,33]. Thus, the observed HDAC inhibitory effect of SF5-SAHA is important in terms of its anticancer potential. In addition, SF5-SAHA showed distinct HDAC6 inhibitory activity which was superior to the activity of SAHA. SF5-SAHA was also slightly more active against HDAC6 than against HDAC1 and HDAC2, an effect, which was also reported of its previously published trifluoromethyl analog [15]. In contrast to most HDACs, HDAC6 is a cytoplasmic non-histone deacetylase enzyme that deacetylates cancer relevant cytoplasmic substrates such as α -tubulin and Hsp90 [34,35]. The HDAC6-based effects on Hsp90 seem to be important in leukemia cells [35]. In prostate cancer cells, deacetylated Hsp90 binds to and stabilizes the androgen receptor (AR), while the HDAC6 suppressor sulforaphane led to increased levels of acetylated Hsp90 accompanied by enhanced AR degradation [36]. In HCC cells, HDAC6 overexpression enhanced migration and invasion activity [37]. Hence, a strong inhibition of HDAC6 by SF5-SAHA can contribute to its anticancer properties, in particular, in the HCC and leukemia cells where SF5-SAHA was more antiproliferative than SAHA.

Our experiments for SF5-SAHA-related effects on EGFR signaling also provided interesting new results. Both SF5-SAHA and SAHA suppressed EGFR expression in DU145 cells, indicating that HDAC inhibition also modulates EGFR levels in cancer cells. The distinct suppression of activated/phosphorylated mTOR in DU145 cells treated with SF5-SAHA is of particular interest. EGFR activates PI3K/Akt signaling with mTOR as downstream factor, rendering mTOR a valuable anticancer target. However, the efficacy of mTOR inhibitors suffers from drug-induced Akt activation. SAHA in combination with the mTOR inhibitor ridaforolimus showed synergistic effects in sarcoma cells based on the suppression of Akt phosphorylation by SAHA [38]. This promising drug combination passed a phase I clinical trial with remarkable outcome in advanced renal cell carcinoma patients [39]. SF5-SAHA also showed antiangiogenic effects in the CAM assay similar to those by SAHA as previously described by our groups [16]. The antiangiogenic activity of SAHA is based on its interference with VEGFR signaling pathways [40]. However, mTOR suppressors also blocked angiogenesis [41]. Thus, the mTOR suppression in DU145 prostate cancer cells treated with SF5-SAHA might be a mechanism of this new compound preventing angiogenesis, too.

Considering the described antiproliferative activities and anticancer modes of action, SF5-SAHA has the potential to overcome SAHA irresponsiveness in certain cancer types. SAHA-resistant entities, which are sensitive to ROS, can be susceptible to a treatment with SF5-SAHA [25]. Distinct HDAC6 inhibition can also sensitize cancer cells to SAHA treatment because the HDAC6-selective inhibitor tubacin was able to increase apoptosis and DNA damage induced by SAHA in LNCaP prostate tumor cells [42].

4. Materials and Methods

4.1. General Chemical Procedures and Materials

Starting materials and reagents were purchased from Sigma-Aldrich (Thermo Fisher Scientific, Waltham, MA, USA). The following instruments were used: IR spectra, Perkin-Elmer Spectrum One FT-IR spectrophotometer with ATR sampling unit; nuclear magnetic resonance spectra, BRUKER Avance 300 spectrometer; chemical shifts are given in parts per million (δ) downfield from tetramethylsilane as internal standard; mass spectra, Varian MAT 311A (EI); Thermo Fischer Scientific Q Exactive (ESI-HRMS); Elementar Unicube (EA).

4.2. Synthesis of SF5-SAHet

4-(Pentafluorothio)aniline (53 mg, 0.24 mmol) was dissolved in dry CH_2Cl_2 and ethyl hydrogen suberate (49 mg, 0.24 mmol), EDCI (71 mg, 0.37 mmol), DMAP (14 mg, 0.095 mmol), and triethyl amine (172 μL , 0.78 mmol) were added. After stirring at rt for 24 h, the solvent was evaporated and the residue was purified by column chromatography (silica gel 60). The obtained residue was dissolved in ethyl acetate and washed with saturated $\text{NH}_4\text{Cl}/1\text{ M HCl}$ (1:1) to remove further impurities. The organic phase was washed with water, dried over Na_2SO_4 , filtered and the filtrate was concentrated in vacuum. Yield: 61 mg (0.15 mmol, 63%); colorless gum; $R_f = 0.37$ (ethyl acetate/*n*-hexane, 1:2); ν_{max} (ATR)/ cm^{-1} 3344 (NH), 2946 (CH), 2867 (CH), 1699 (CO), 1630 (CO), 1596, 1533, 1501, 1468, 1419, 1403, 1381, 1333, 1310, 1258, 1232, 1186, 1166, 1098, 1037, 1009, 836, 810, 732, 694, 669, 632; $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 1.2–1.4 (7 H, m), 1.5–1.7 (4 H, m), 2.2–2.4 (4 H, m), 4.09 (2 H, q, $J = 7.1$ Hz), 7.6–7.7 (4 H, m), 7.90 (1 H, s); $^{13}\text{C NMR}$ (75.5 MHz, CDCl_3) δ 14.2, 24.6, 25.1, 25.3, 28.5, 34.1, 37.4, 60.4, 113.3, 118.9, 126.8, 126.9, 127.0, 127.2, 127.4, 127.5, 140.8, 148.5, 148.7, 149.0, 149.2, 149.4, 171.8, 174.0; m/z (%) 403 (13) [M^+], 358 (26), 261 (41), 219 (100), 185 (36), 111 (25), 83 (25).

4.3. Synthesis of SF5-SAHA

SF5-SAHet (61 mg, 0.15 mmol) was dissolved in $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (9 mL, 1:2), hydroxylamine (50% in water, 0.5 mL, 15 mmol) and NaOH (200 mg, 5 mmol) were added and the reaction mixture was stirred at rt for 1 h. The solvent was removed, the residue was dissolved in water and adjusted to pH 8 with acetic acid. The aqueous phase was extracted with ethyl acetate (2 \times 50 mL), dried over Na_2SO_4 and concentrated in vacuum. The solid residue was recrystallized from $\text{CH}_2\text{Cl}_2/n$ -hexane. Yield: 50 mg (0.13 mmol, 87%); colorless gum; ν_{max} (ATR)/ cm^{-1} 3247 (NH), 2951 (CH), 2864 (CH), 2405 (br, OH), 1665 (CO), 1647 (CO), 1621, 1597, 1538, 1505, 1469, 1447, 1401, 1352, 1311, 1264, 1226, 1203, 1101, 1061, 1040, 999, 968, 939, 832, 803, 762, 734, 668, 632; $^1\text{H NMR}$ (300 MHz, MeOD) δ 1.3–1.4 (4 H, m), 1.6–1.8 (4 H, m), 2.0–2.2 (2 H, m), 2.3–2.5 (2 H, m), 7.7–7.8 (4 H, m); $^{13}\text{C NMR}$ (75.5 MHz, MeOD) δ 26.6, 30.1, 33.8, 38.0, 120.3, 127.9, 128.0, 128.1, 149.7, 150.0, 150.2, 173.0, 175.1; HRMS (ESI) m/z [$\text{M} + \text{H}^+$] calcd. for $\text{C}_{14}\text{H}_{20}\text{F}_5\text{N}_2\text{O}_3\text{S}^+$ 391.11093, found: 391.10977; Anal. calcd. for $\text{C}_{14}\text{H}_{19}\text{F}_5\text{N}_2\text{O}_3\text{S}$: C, 43.08, H, 4.91. Found: C, 43.20, H, 4.96.

4.4. Biological Evaluations

4.4.1. Cell Culture

Human Hep-G2 cells (ATCC No HB-8065) and DU145 cells (ATCC No HTB-81) were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI) supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, 50 U/mL penicillin-streptomycin (all from Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and grown in an incubator (37 $^\circ\text{C}$, 5% CO_2 , humidified atmosphere). Jurkat and SupT11 cell lines were purchased from DSMZ (Braunschweig, Germany). Hut78 cells were purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany) and the SMZ-1 cell line was provided by Dr. Raphael Koch from the University Göttingen. The T-cell leukemia/lymphoma cell lines were cultured in RPMI-1640 medium including L-glutamine (Gibco) supplemented with 10% or 20% fetal bovine serum (Sigma-Aldrich, Darmstadt, Germany) and penicillin/streptomycin

(100 U/0.1 M) at 37 °C in a 5% CO₂ incubator with 95% humidity. The reference compound SAHA was purchased from Sigma-Aldrich (Darmstadt, Germany).

4.4.2. Antiproliferative Activity Assay

Stock solutions (10 mM) prepared by dissolution in DMSO were stored at −20 °C. Working solutions were always freshly prepared before each experiment by dilution of stock solution with medium. The final DMSO concentration never exceeded 0.25%. Treatment-induced changes in cell number were determined by crystal violet (N-hexamethylpararosaniline from Sigma Aldrich) staining [32]. 1000 cells/well (DU145) or 1500 cells/well (Hep-G2) were seeded in 96-well plates and maintained for adherence in an incubator (37 °C, 5% CO₂, humidified atmosphere) for 48 h prior to the beginning of the treatment. Subsequently, cells were treated with rising concentrations (0–10 μM) of the novel compounds, or SAHA, respectively, for 72 h. Thereafter, the cells were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet. The unbound dye was removed by rinsing with water. Bound crystal violet was solubilized with 0.2% Triton X-100 (Sigma-Aldrich). Light extinction of crystal violet, which increases linearly with the cell number, was analyzed at 570 nm using an ELISA-Reader (Dynex Technologies, Denkendorf, Germany). For T-cell leukemia/lymphoma cell lines apoptosis was determined using dual staining for Annexin-V and 7AAD via flow cytometry. Time- and dose-dependent growth inhibition as well as IC₅₀ values are given as means ± SEM of n ≥ 3 independent experiments performed in triplicates or more.

For T-cell leukemia/lymphoma cell lines, IC₅₀ values were determined using dual staining for Annexin-V and 7AAD via flow cytometry [17].

4.4.3. Determination of Unspecific Cytotoxicity

To exclude unspecific cytotoxicity as the driving mode of action for antiproliferative effects, the release of lactate dehydrogenase (LDH) from DU145 cells was determined after 3 h and 24 h of treatment using the Cytotoxicity Detection Kit PLUS LDH (Roche Diagnostics GmbH, Mannheim, Germany). The assay was performed as described earlier [43]. Cytotoxicity was determined by subtracting the percentage of LDH release into the supernatant under control conditions of those from treated samples. Measurements were performed in duplicate in n = 3 independent experiments and mean percentage changes ± SEM as compared to controls are shown.

4.4.4. Apoptosis-Specific Caspase-3 Activation

Changes in caspase-3 activity were measured by the cleavage of the fluorogenic substrate AC-DEVD-AMC (EMD Millipore, Billerica, MA, USA), as described previously [43]. After incubation with SF5-SAHA or SAHA (1–10 μM) for 24 h the cells were harvested and lysed with lysis buffer. Subsequently, the lysates were incubated for 1 h at 37 °C with a substrate solution containing 20 μg/mL AC-DEVD-AMC, 20 mM HEPES, 10% glycerol and 2 mM DTT at pH 7.5. Substrate cleavage was measured fluorometrically using a Varioskan Flash fluorometer (Thermo Fisher Scientific, Waltham, MA, USA; filter sets: ex 360/40 nm, em 460/10 nm). n = 3 independent measurements were performed in triplicate, and data are given as the mean percentage increase ± SEM above control, which was set at 100%.

4.4.5. ROS Formation

The formation of cytosolic ROS in DU145 and Hep-G2 cells after treatment with SF5-SAHA or SAHA (10 μM) was measured using the membrane permeable dye CellROX[®] Orange (Thermo Fisher Scientific) which accumulates in the cytoplasm, exhibiting strong fluorescent signals at excitation/emission levels of 545 nm/565 nm upon oxidation [44]. Untreated cells incubated with 1.6 mM H₂O₂ for 30 min served as positive controls. CellROX[®] Orange reagent (1 μM) was applied, when adding the test compounds. Formation of ROS was measured after 3 h, 6 h, 12 h (not shown) and 24 h using ZOE[™] Fluorescent Cell

Imager (Biorad, Munich, Germany). $n = 3$ independent experiments performed in triplicate for each condition.

4.4.6. HDAC Inhibition

The HDAC-inhibitory potential of SF5-SAHA was determined by using fluorogenic HDAC Assay kits for the detection of pan-HDAC activity (Calbiochem, Merck Chemicals, Darmstadt, Germany) or the subtype specific HDAC1, HDAC2 and HDAC6 activity (BPS Biosciences, San Diego, CA, USA). HDAC activity was measured according to the instructions of the supplier. SAHA (1–10 μM) served as positive control. Human HDAC enzymes derived from HeLa cell nuclear extracts (pan-HDAC assay) or human recombinant HDAC1, HDAC2 and HDAC6 enzymes and adjacent fluorogenic HDAC substrates were used to determine HDAC activity levels. A 50 μL assay buffer containing 1 $\mu\text{g}/\mu\text{L}$ bovine serum albumin, the human HDAC enzymes, SF5-SAHA (1–10 μM) and the corresponding HDAC substrates were added into a black 96-well assay plate. The reaction in each well was incubated at 37 $^{\circ}\text{C}$ for 30 min, followed by adding 50 μL HDAC developer reagent, and incubated at room temperature for an additional 15 min. Fluorescence intensity of the assay plates was measured on a Varioskan Flash fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using excitation wavelengths of 350 nm to 380 nm and an emission wavelength of 460 nm. Docking studies of SF5-SAHA and SAHA bound to the active site of HDAC2 (PDB ID 4LXZ) were carried out using AutoDock Vina in the presence of the catalytic Zn^{2+} ion at the binding site. The co-crystallized ligands and the Zn^{2+} ion were used as reference to define the binding pockets within a radius of 30 \AA . The protein preparation and ligand preparation procedures were done using the open-source web server DockThor (www.dockthor.lncc.br, accessed on 19 October 2021) and Merck molecular force field was applied. The protein preparation was done at physiological pH 7.4. All dockings and calculations were performed using AutoDock-Vina 1.1.2 software [45]. All other settings for the ligand and receptor definitions were used as default. The docking strategy, scoring and chemical parameters were kept as default. For each compound, nine poses were generated, and all were evaluated with the built-in scoring function. PyMOL software was utilized to visualize, compare and analyze the binding pose predictions, and to create the images [46]. For visualization, the protein was used in the cartoon mode and the surface mode, wherein the metal chelation of Zn^{2+} was visualized in the sphere mode and nb sphere mode, respectively (Figure 9).

4.4.7. Western Blots

Western blots were performed as described earlier [47]. Cells were seeded in 100 mm petri dishes, grown to almost confluency, treated for 24 h, washed and frozen, followed by lysis with RIPA Buffer, added with one cComplete™ Mini protease Inhibitor tablet/10 mL (Roche), and quantification with Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Thereafter protein levels were normalized to untreated controls so equal protein loading of 20 $\mu\text{g}/\text{lane}$. Laemmli Buffer and β -mercaptoethanol were added before probes were desaturated at 96 $^{\circ}\text{C}$ for 10 min. First, 7.5% or 12% SDS gels (Biorad, Munich, Germany) were loaded with proteins and electrophoresis was done. Thereafter, proteins were transferred to activated polyvinylidene difluoride membranes (PVDF) by electroblotting. Finally, the membranes were blocked with 1% BSA and incubated with primary antibodies over night at 4 $^{\circ}\text{C}$. The following antibodies acetylated histone H3 (ab47915 Abcam, 1:1000), acetyl- α -tubulin (5335, Cell Signaling, 1:1000), EGFR (sc03 Santa Cruz Biotechnology, 1:500), poly-(ADP-ribose)-polymerase (PARP) and cleaved PARP (11835238 Roche, 1:1000), Apaf-1 (ab5088 Abcam 1:500), Bcl-2 (7382 Santa Cruz Biotechnology, 1:1000) and β -actin (A5441 Sigma Aldrich, 1:2000) for standardization were used. levels also decreased in a dose-dependent way in three out of four cell lines. Only the Jurkat cells had already a high level of α -tubulin in untreated cells and, thus, drug-induced changes were not detectable in these cells.

Blots were washed three times with 1% TBS-Tween and incubated with anti-mouse or anti-rabbit peroxidase-coupled anti-IgG secondary antibodies (1:5000–1:10,000) at room temperature for 60 min. Subsequently, antibody bondage was illustrated using Clarity and Clarity Max ECL Western Blotting Substrates (Biorad, Munich, Germany) for detection and Celvin-S developer (Biostep, software SnapAndGo Vs 1.8.1) for development. Independent blots of $n = 3$ experiments were generated, and the expression levels were determined by analyzing the bands with ImageJ and calculating the area under the curve (AUC) relative to its greyscale. Values were normalized to β -actin expression, which served as a loading control and compared to untreated control expressions which was set at 1. The mean \pm SEM of the relative values to control are illustrated with Graph Pad Prism 8.

4.4.8. Scratch Assay

To investigate the anti-migratory effects of SF5-SAHA scratch assays were performed as described [48]. In brief, cells were grown to sub-confluency in 6-well plates. The cell monolayer was then scratched using a pipet tip. Cells at the edge of this artificial gap migrate into the cell-free area to close the gap in a time-dependent manner. DU145 cells were seeded at a density 1.5×10^5 cells/well and treated with SF5-SAHA (0–10 μ M) for 24 h and bright-field images were taken before and after 12 h and 24 h of treatment using an EVOS M5000 microscope (Thermo Fischer Scientific, Waltham MA, USA). The experiments were performed $n = 3$.

4.4.9. In Vivo/Ovo Evaluation of Angiogenesis

Anti-angiogenesis activity of SF5-SAHA was tested on the CAM (chorioallantoic membrane) of fertilized chicken eggs [49]. The embryonic development was started by placing the eggs in a humidified (>60%) incubator in an upright position at a temperature of 37.8 °C. After 3 days, a hole of 2 mm was pierced in the eggshell top side in order to detach the developing CAM from the eggshell and let it sink into the allantoic cavity. After 10 days, the eggshell hole was broadened, and a silicone ring (1 cm \varnothing) was placed on the CAM, which was allowed to attach for 6 h. Then, 20 μ L of SF5-SAHA containing medium or PBS (control) was added into the ring. The angiogenic states of the CAM were documented using a stereomicroscope equipped with a Kappa digital camera system (Distelkamp-Electronic, Kaiserslautern, Germany). Pictures were taken every 24 h until the finish of the experiment after 72 h. The CAM experiments were carried out with $n = 3$ eggs for each condition.

4.4.10. Statistical Analysis

Statistical calculations were carried out with GraphPad Prism 8 (GraphPad Software Vs 8.2.1, San Diego, CA, USA) using 2-way ANOVA Dunnett's post-hoc test for statistical significance testing or linear regression.

5. Conclusions

The synthesis and anticancer modes of action of a new SAHA analog with a *para*-pentafluorosulfanyl substituted phenyl ring were described. The compound was found to encompass pronounced antiproliferative, apoptosis inducing and ROS forming effects in a panel of hematological and solid cancer cell models, warranting further studies to elucidate the underlying mechanisms and suitability of this promising compound as an anticancer drug candidate in the future.

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administration, M.H. (Michael Höpfner) and B.B. All authors have read and agreed to the published version of the manuscript.

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Article

Novel Thienyl-Based Tyrosine Kinase Inhibitors for the Treatment of Hepatocellular Carcinoma

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Abstract: New medical treatments are urgently needed for advanced hepatocellular carcinoma (HCC). Recently, we showed the anticancer effects of novel thiophene-based kinase inhibitors. In this study, we further characterized the antineoplastic effects and modes of action of the two most promising inhibitors, Thio-Iva and Thio-Dam, and compared their effects with the clinically relevant multi-kinase inhibitor, sorafenib, in HCC cells. Crystal violet staining and real-time cell growth monitoring showed pronounced antiproliferative effects in Huh-7 and SNU-449 cells with IC₅₀ values in the (sub-)micromolar range. Long-term incubation experiments revealed the reduced clonogenicity of Thio-Iva and Thio-Dam-treated HCC cells. LDH-release tests excluded cytotoxicity as an unspecific mode of action of the inhibitors, while flow cytometry analysis revealed a dose-dependent and pronounced G2/M phase cell cycle arrest and cyclin B1 suppression. Additionally, mitochondria-driven apoptosis was observed through the cytosolic increase of reactive oxygen species, a concomitant PARP cleavage, and caspase-3 induction. Both compounds were found to effectively inhibit the capillary tube formation of endothelial EA.hy926 cells in vitro, pointing towards additional antiangiogenic effects. Antiangiogenic and antineoplastic effects were confirmed in vivo by CAM assays. In summary, the thienyl-acrylonitrile derivatives, Thio-Iva and Thio-Dam, exert significant antineoplastic and antiangiogenic effects in HCC cells.

Keywords: hepatocellular carcinoma; anticancer drugs; treatment; angiogenesis; multi-kinase inhibitor



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1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world [1]. HCC emerges in patients with chronic liver inflammation associated with viral infection, alcohol abuse, or metabolic syndrome. Its incidence is constantly rising, and the relative 5-year survival rate is below 20%. At present, the clinical treatment options for early-stage HCC include surgical resection, liver transplantation, or percutaneous ablation [3]. However, as most patients are already in an advanced disease stage when diagnosed, these radical treatment options are often not applicable. For patients with advanced HCC, palliative therapy by trans-arterial chemoembolization or systemic therapy with tyrosine kinase inhibitors such as sorafenib are methods of choice. However, tumor growth control, relieve of disease-related symptoms, and the overall survival of sorafenib-treated patients with advanced HCC is not convincingly improved and is hampered by the occurrence of resistance to sorafenib treatment [4]. Thus far, no effective medical treatment exists for patients suffering from advanced HCC, emphasizing the urgent need for new and efficient therapeutic agents for HCC treatment.

Key enzymes of cellular signal transduction pathways correlated with tumor cell differentiation and proliferation are valuable targets for anticancer drug screening leading

to the development of new drug candidates with high efficiency, low toxicity, and high specificity [5].

Sorafenib is a prominent clinically approved example of a small molecule multi-kinase inhibitor, which targets vascular endothelial growth factor receptors (VEGFR) 1–3, platelet-derived growth factor receptor- β (PDGFR- β), and rapidly accelerated fibrosarcoma kinases (Raf kinases) [6]. Sorafenib was the only first-line systemic targeted drug available for advanced HCC for almost one decade, with a survival benefit of three months [7].

However, clinical studies have reported that a considerable proportion of HCC patients does not respond to sorafenib treatment. The response rate to sorafenib is less than 50%, and most patients develop disease progression within six months [8,9]. Due to the early occurrence of sorafenib resistance, most patients do not have long-term benefits, and thus the overall efficacy of sorafenib is far from satisfactory. Over the last years, further first-line and second-line therapies which are based on the receptor tyrosine kinase inhibitors regorafenib, lenvatinib, or ramucirumab have emerged [10–12]. Immunotherapy is a relatively new field of HCC research. The majority of HCCs arise from chronic liver disease where T cells are constantly exposed to antigen and inflammatory signals. This condition induces a state of upregulated receptors, such as the programmed cell death protein-1 (PD-1). The PD-1 inhibitor nivolumab was approved in 2017 as a second-line treatment for advanced HCC but failed to show statistically significant benefits over sorafenib such as being progression-free and improved overall survival rates [13]. These drawbacks of HCC immunotherapies necessitate stronger efforts in the search for new drugs against HCC.

Tumor angiogenesis plays a vital role in the growth and dissemination of solid tumors. Hypervascularity and marked vascular abnormalities such as arterialization and sinusoidal capillarization are common tumor-associated features of HCC [14]. Vascular endothelial growth factors (VEGFs, e.g., VEGF-A, VEGF-B, VEGF-C, VEGF-D) and the receptor tyrosine kinases (RTKs) VEGFR-1, VEGFR-2, and VEGFR-3 are crucial for the promotion of tumor angiogenesis [15,16]. Tumor-induced angiogenesis is based on two mechanisms, i.e., the overexpression of angiogenic factors and the inhibition of anti-angiogenic factors, which lead to the enhanced development of blood vessels lacking in normal vascular structures with regulated blood vessel diameter and tissue-related perfusion. Antiangiogenic therapy based on antibodies and small-molecule VEGFR inhibitors was developed to inhibit the growth and further spreading of abnormal tumor blood vessels that lead to tumor hypoxia and shrinkage [17,18].

Various reports have described the promising anti-tumor activities of thiophene-based compounds [19,20]. Recently, we identified some *E*-2-(2-thienyl)-3-acrylonitrile derivatives with high anti-tumor efficacy in p53 wild-type HepG2 hepatoblastoma cells which were more active than the clinically applied multi-kinase inhibitor sorafenib [21]. These compounds are likewise multi-kinase inhibitors with preferential activity against VEGFR-2. In this work, we further analyzed the mode of action of these promising compounds (Figure 1) in HCC cells.

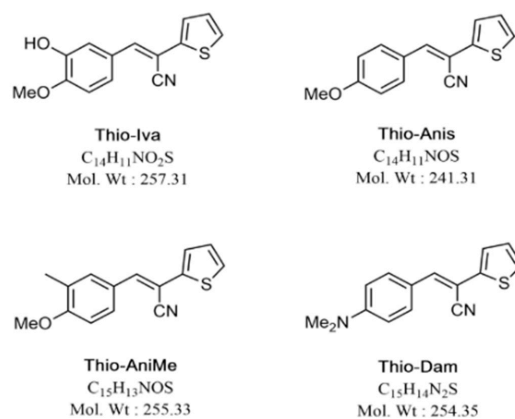


Figure 1. Chemical structures of E-2-(2-thienyl)-3-acrylonitrile RTK inhibitors used in this study.

2. Materials and Methods

2.1. Compounds

Stock solutions (10 mM) of Thio-Iva, Thio-Dam, Thio-Anis, Thio-Anime, and sorafenib were prepared in dimethyl sulfoxide (DMSO Thermo Fisher Scientific, Inc., Waltham, MA, USA) and stored at $-20\text{ }^{\circ}\text{C}$. Sorafenib was purchased from Targetmol (T0093L, Boston, MA, USA). Thio-Iva, Thio-Dam, Thio-Anis, and Thio-Anime were synthesized and provided by Dr. Biersack (Dept. of Organic Chemistry, University of Bayreuth, Bayreuth, Germany) [21].

2.2. Biological Evaluation

2.2.1. Cell Culture

Highly differentiated and p53-mutated Huh-7 (JCRB#0403) human hepatocellular carcinoma cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (all from Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The p53-mutated SNU-449 cells (ATCC#2234) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 1% HEPES, and 1% sodium pyruvate. EA.hy926 human umbilical vein cells were grown in DMEM containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. All cells were incubated at $37\text{ }^{\circ}\text{C}$, 5% CO_2 , 95% humidified atmosphere.

2.2.2. Crystal Violet Staining

The treatment-induced inhibition of cell proliferation was assessed using crystal violet staining, as described earlier [22]. In brief, 1500 cells/well seeded in 96-well plates were allowed to adhere to the bottom of the wells for 72 h. Thereafter, the cells were incubated with rising concentrations (0.1–20 μM) of each test compound for up to 48 h. After that, cells were rinsed with PBS, fixed with 1% glutaraldehyde, and 0.1% crystal violet (N-hexamethylpararosaniline, Sigma Aldrich) was added to stain the cells. Unbound dye was removed by rinsing with water. The cell-bound crystal violet was dissolved using 0.2% Triton X-100 (Sigma-Aldrich, Munich, Germany). The extinction of crystal violet, which increases linearly with the increase of the cell number, was measured with an ELISA-Reader (Dynex Technologies, Denkendorf, Germany) at 570 nm [23].

2.2.3. Real-Time Monitoring of Cell Proliferation

The real-time cell analyzer iCELLigence system (ACEA Biosciences San Diego, CA, USA) was used to monitor cell proliferation and survival, as previously described [24]. Cells were seeded in 8-well micro-E-plates (ACEA Biosciences, San Diego, CA, USA) at a

density of 6000 cells/well. Incubation for 24 h allowed for attachment, and the medium was replaced thereafter by Thio-Iva or Thio-Dam-containing medium in rising concentrations (0.1–10 μM). The impedance-based iCELLigence system determined proliferation by measuring changes in the electrical resistance of the bottom of each well every 15 min, for up to 96 h. Electrical resistance increases when the number of attached cells increases due to mitosis. Data are recorded as a unitless parameter called cell index, which is defined as $(R_{t_n} - R_{t_0}) / 4.6 \text{ Ohm}$, with R_{t_n} being the measured resistance at time point n and R_{t_0} being background resistance measured at time point T_0 .

2.2.4. Colony Formation Assay

The proliferation of long-term effects was assessed by colony formation assays. Cells were seeded in 6-well plates at a density of 300 cells/well, and colony formation and growth were observed for 2 weeks [21]. Then, the colonies were washed twice with PBS and fixed with 4% formaldehyde for 1 h before staining with 0.5% crystal violet for 3 min. A colony was defined as a cell aggregate with 50 or more cells [21], and so only colonies with 50 or more cells were counted. Representative images were taken by a kappa digital camera system (Kappa Optronics GmbH, Gleichen, Germany). Stained colonies were quantified using the Colony Area ImageJ plug-in application (Vision 1.52a, National Institutes of Health, USA).

2.2.5. Enzymatic Kinase Assay

A cell-free kinase assay consisting of a custom panel of 32 protein kinases involved in cell proliferation and angiogenesis was used to screen the kinase-inhibiting potency of Thio-Iva (10 μM). The assay was performed by Eurofins Kinase Profiler TM service (Eurofins, Celle-Lévescault, France), and the determination of enzymatic activity was assessed as previously described [21]. Moreover, a dose-response curve for Thio-Iva (0.003–30 μM) was executed to determine the IC_{50} of Thio-Iva-induced VEGFR-2 inhibition.

2.2.6. Determination of Caspase-3

Caspase-3 activity was measured to determine Thio-Iva and Thio-Dam-induced apoptosis in HCC cells [25]. A total of 100,000 cells/well were seeded in 6-well plates and maintained for 24 h. Thereafter, the cells were incubated with 1 μM and 10 μM of each test compound for 24 h and 48 h, respectively. After that, the cells were collected and lysed using lysis buffer at 4 $^{\circ}\text{C}$ for 30 min. The protein content of the samples was quantified using PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific) to adjust equal amounts of protein for the following caspase-3 determination. The samples were incubated with AC-DEVD-AMC (EMD Millipore, Billerica, MA, USA) at 37 $^{\circ}\text{C}$ for 1 h. Active caspase-3 cleaved AC-DEVD-AMC to produce fluorescent AC-DEVD, which was measured using a VarioScan Flash 40053 microplate luminometer (Thermo Fisher Scientific, Waltham, MA, USA; filter sets: e.g., 360/40 nm, em 460/10 nm) [21].

2.2.7. Determination of Compound-Induced Cytotoxicity

Cytotoxicity was quantified with a KitPLUS lactate dehydrogenase (LDH) assay (Roche Diagnostics GmbH, Mannheim, Germany). A total of 4×10^4 cells/well were seeded in 96-well plates for 24 h and then incubated with 1 μM and 10 μM of Thio-Iva and Thio-Dam for 6 h and 24 h. Supernatant was collected for LDH determination according to the manufacturer's instructions. Then, 100 μL of a mixture of catalyst and dye solution was added, and cells were incubated for a maximum of 30 min. LDH can catalyze the synthesis of pyruvate from lactic acid, and then the reaction of pyruvate to 2,4-dinitrophenylhydrazine, which forms a brownish red solution under basic conditions. An ELISA reader (Dynex Technologies, Denkendorf, Germany) was used at 490/630 nm for the measurement of cytotoxicity indicating the leakage of LDH into the supernatant of the cells. Data are expressed as the percentage (%) of the total LDH activity (LDH in the

medium + LDH in the cell), according to the equation % LDH released = (LDH activity in the medium/total LDH activity) \times 100 [23].

2.2.8. Scratch Assay

Cells (1.5×10^5 cells/well) were seeded in 6-well plates and allowed to grow to (sub-) confluence. Using a 10 μ L pipette tip, the cell monolayer was scratched vertically. The cells on the edge of this artificial gap migrate into the cell-free area to close the gap in a time-dependent manner. The cells were rinsed with PBS, and fresh medium was added, which contained rising concentrations of Thio-Iva and Thio-Dam (1–10 μ M), and a corresponding volume of DMSO was used for control. The cells were incubated for 24 h (37 $^{\circ}$ C, 5% CO₂, 95% humidity), followed by photographic documentation with an EVOS M5000 microscope (Thermo Fischer Scientific, Waltham, MA, USA). The migration of cells was quantified using the TScratch software (<https://github.com/cselab/TScratch>, accessed on 5 April 2022; CSElab, Zurich, Swiss). Migration values were normalized to control, which was set as 100% [25].

2.2.9. Measurement of Reactive Oxygen Species (ROS)

The formation of cytosolic ROS was performed as described [26]. Measurement was performed by using the membrane-permeable dye CellROX[®] Orange (Thermo Fisher Scientific, Inc., Waltham, MA, USA), which accumulates in the cytoplasm and exhibits a strong fluorescent signal upon oxidation at excitation/emission levels of 545 nm/565 nm [26]. CellROX[®] Orange reagent (1 μ M) was applied together with Thio-Iva (1 and 10 μ M) and Thio-Dam (1 and 10 μ M). Cells incubated with 1.6 mM H₂O₂ for 30 min served as positive controls. Formation of ROS was measured after 24 h of incubation with the compounds using a ZOE[™] Fluorescent Cell Imager (Biorad, Munich, Germany).

2.2.10. Cell-Cycle Analysis by Flow Cytometry

Flow cytometry was applied for cell cycle analysis by staining the DNA of treated HCC cells with propidium iodide (PI) nucleic acid stain (Invitrogen, Eugene, OR, USA). Huh-7 and SNU-449 cells were seeded in 6-well plates with a density of 20,000 cells/mL and treated with different concentrations of Thio-Iva and Thio-Dam for 48 h. Then, the cells were harvested and fixed in 70% ethanol overnight and incubated with RNaseA (0.4 mg/mL) in PBS at 37 $^{\circ}$ C for 30 min. PI was added and cells were incubated for 30 min in the dark. Samples were analyzed using FACSCanto II (BD Biosciences, Heidelberg, Germany). Data analysis was done with FlowJo 10.4 software (LLC, Ashland, OR, USA).

2.2.11. Tube Formation

A total of 50 μ L of cold Matrigel/well (Corning[™] 354234, Tewksbury, MA, USA) was plated out in a 4 $^{\circ}$ C cold 96-well plate and allowed to polymerize in a 37 $^{\circ}$ C incubator for 2 h. Then, EA.hy926 cells were suspended in DMEM containing different concentrations of Thio-Iva and Thio-Dam and inoculated to the Matrigel at a density of 2500 cells/well. After incubation at 37 $^{\circ}$ C and 5% CO₂ for 6 h, photographic documentation was executed with an EVOS M5000 microscope (Thermo Fischer Scientific, Waltham, MA, USA). For the analysis and quantification of tube formation, the Angiogenesis Analyzer plugin of ImageJ (NIH, Bethesda, MD, USA) was employed. Results are expressed as total segment length.

2.2.12. Western Blot

Western blotting was performed as described previously [27]. In short, radioimmunoprecipitation assay (RIPA) buffer was used to lyse whole-cell extracts. Protein was quantified by the bicinchoninic acid (BCA) assay. An equal amount of protein (20 μ L) was separated from each sample by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (PVDF), followed by incubation with primary antibody overnight at 4 $^{\circ}$ C. Antibodies of phospho-VEGF Receptor2 (#3817 Cell Signaling Technology, Danvers, MA, USA, 1:500), cyclin B1 (sc-245 Santa Cruz Biotech-

nology, Santa Cruz, CA, USA, 1:1000), poly-(ADP-ribose)-polymerase (PARP) and cleaved PARP (11835238 Roche Mannheim, Germany, 1:1000), and β -actin (A5441 Sigma Aldrich, Taufkirchen, Germany, 1:2000) were used. Then, incubation with the corresponding anti-mouse (NA931VS Santa Cruz Biotechnology, 1:10,000) or anti-rabbit (NA934VS Santa Cruz Biotechnology, 1:10,000) peroxidase-coupled anti-IgG secondary antibodies was carried out at room temperature for a minimum of 1 h. Subsequently, antibody bondage was illustrated using Clarity Max ECL Western Blotting Substrates (Biorad, Munich, Germany) for detection and Calvin-S developer (Biostep, software SnapAndGo, Burkhardtendorf, Germany) for development.

2.2.13. Chicken Chorioallantoic Membrane Assay (CAM)

In vivo assays employing the chorioallantoic membrane (CAM) of fertilized chicken eggs were performed to test the anti-neoplastic and anti-angiogenic effects of the novel compounds, as described previously [21]. In short, fertilized chicken eggs (*Gallus gallus*) were obtained from a commercial provider (Valo Biomedica GmbH, Osterholz-Scharmbeck, Germany), and the development was induced by incubating the eggs at a temperature of 37.8 °C with 66% relative humidity. On day 3, the eggs were opened by cutting the shell at the top part of the egg.

For anti-angiogenesis testing, a silicone ring (diameter: 5 mm) was placed on the CAM for 24 h to be able to stably connect the ring to the CAM. On day 12, 20 μ L of test compounds (0.2, 0.5, 1.0 μ M) diluted with 0.9% NaCl was pipetted into the ring. The blood vessel status of the CAM was documented after 48 h by using a digital camera (Distelkamp-Electronic, Kaiserslautern, Germany). The degree of angiogenesis was quantified by measuring the length of the blood vessels using Image Pro Plus 6.0 (Image-pro Plus, Media Cybernetics, Inc., Silver Spring, MD, USA).

For anti-neoplastic testing, a total of 3×10^6 Huh-7 cells were resuspended in 10 μ L cell culture media and 10 μ L Matrigel (Corning™ 354234, MA, Tewksbury, USA) (BD Biosciences) before the cell suspension was applied to a silicone ring (5 mm in diameter) on the CAM of fertilized chicken eggs on day 8 of their embryonic development. The tumor-bearing chicken eggs were incubated for 24 h at 37.8 °C to stimulate tumor formation, followed by the topical application of 20 μ L medium containing Thio-Iva, Thio-Dam, or sorafenib. After an incubation period of 72 h at 37.8 °C and 66% humidity, the tumors were excised and carefully weighed to determine their mass.

2.2.14. Statistical Analysis

GraphPad (version 8.00, San Diego, CA, USA) was used for statistical analysis. Unless otherwise specified, all experiments were independently repeated for 3–5 times, and the results are expressed as means \pm SD or SEM, respectively. Statistical significance was calculated by performing a one-way analysis of variance (ANOVA).

3. Results

3.1. Biological Evaluation

3.1.1. IC₅₀ Determination of Novel Thiophene-Based Compounds in HCC Cells

The crystal violet staining method was used to determine the growth inhibitory effects of the four thiophene-based test compounds on the two human HCC cell lines, Huh-7 and SNU-449. Thio-Iva showed the highest activities, with IC₅₀ values of 0.29 ± 0.18 μ M (Huh-7) and 0.53 ± 0.32 μ M (SNU-449) after 48 h of treatment. Thio-Dam also showed considerable antiproliferative activity, with IC₅₀ values of 0.81 ± 0.26 μ M and 1.64 ± 0.51 μ M, respectively, and thus, both Thio-Iva and Thio-Dam were distinctly more active than the clinically approved VEGFR inhibitor sorafenib (IC₅₀ = 2.50 ± 0.14 μ M for Huh-7 and >8 μ M for SNU-449) (Table 1). The closely related derivatives Thio-Anis and Thio-AniMe were also slightly more active against Huh-7 cells than sorafenib. However, in the SNU-449 cells, Thio-Anis, Thio-AniMe, and sorafenib were distinctly less active and did not show pronounced

antiproliferative activity at doses of 8 μM and higher. Thus, for further evaluations, only the two best working compounds, Thio-Iva, and Thio-Dam, were chosen.

Table 1. Determination of IC_{50} values (μM) of test compounds for the Huh-7 and SNU-449 HCC cell lines after incubation for 48 h.

Compounds	Huh-7	SNU-449
Thio-Iva	0.29 ± 0.18	0.53 ± 0.32
Thio-Dam	0.81 ± 0.26	1.64 ± 0.51
Thio-Anis	1.20 ± 0.42	>8
Thio-AniMe	1.85 ± 0.21	>8
Sorafenib	2.50 ± 0.14	>8

3.1.2. Kinase Inhibitory Effects of Thio-Iva and Thio-Dam

A cell-free kinase assay was used to screen for the kinase inhibitory potency of the most effective thiophene-based test compound, Thio-Iva, in a customized panel of 32 protein kinases. The kinases were selected because of their relevance to the proliferation and angiogenesis of HCC [21]. Thio-Iva showed multi-kinase inhibitory activity, with the most pronounced effects on VEGFR-2, which was inhibited by $\sim 90\%$ (Figure 2a). In the following step, a dose–response curve was determined for the inhibition of VEGFR-2 by Thio-Iva (0.003–30 μM) (Figure 2b). In the cell-free kinase assay, the IC_{50} value of Thio-Iva amounted to 3.31 μM .

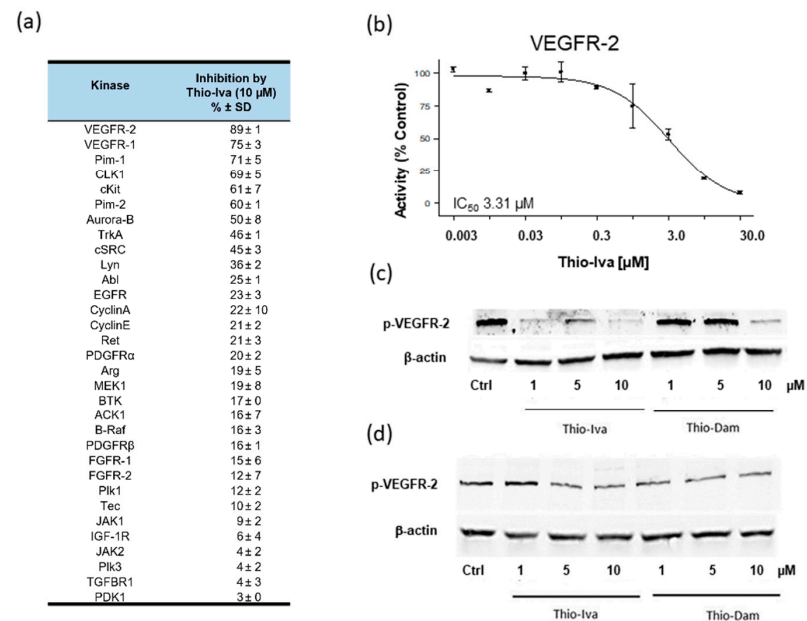


Figure 2. VEGFR-2 inhibition by novel compounds. Enzymatic kinase profiling on 32 kinases revealed multi-kinase inhibition of Thio-Iva, with the most pronounced effects on VEGFR-2 kinase (a). Dose–response curve for Thio-Iva-induced VEGFR-2 kinase inhibition (b). Data are given as means \pm SD of $n = 2$ –3 independent determinations per kinase. Western blot of Thio-Iva and Thio-Dam-induced inhibition of VEGFR-2 phosphorylation in Huh-7 (c) and SNU-449 (d) cells after 24 h of incubation, confirming the VEGFR-2 inhibiting effects of both compounds on the cellular level.

To confirm VEGFR-2 inhibiting effects on the cellular level, the dephosphorylating effects of Thio-Iva and Thio-Dam were determined in HuH-7 and SNU-449 cells (Figure 2c,d). Both compounds showed a dose-dependent suppression of VEGFR-2 phosphorylation in both cell lines, with Thio-Iva being more effective as compared to Thio-Dam.

3.1.3. Antiproliferative Activity of Thio-Iva and Thio-Dam in HCC Cells

In order to further evaluate the dynamic effects of Thio-Iva and Thio-Dam on the proliferation of HCC, the iCELLigence system monitoring cell proliferation in real time was used. In the control group, both Huh-7 (Figure 3a) and SNU-449 (Figure 3c) cells showed vigorous growth, as shown by the cell index (CI), which continued to increase over time. By contrast, when treated with Thio-Iva or Thio-Dam (0.1–1.0 μM), both cell lines showed a dose-dependent reduction in the increase of the CI values, indicating a dose-dependent reduction of cell proliferation.

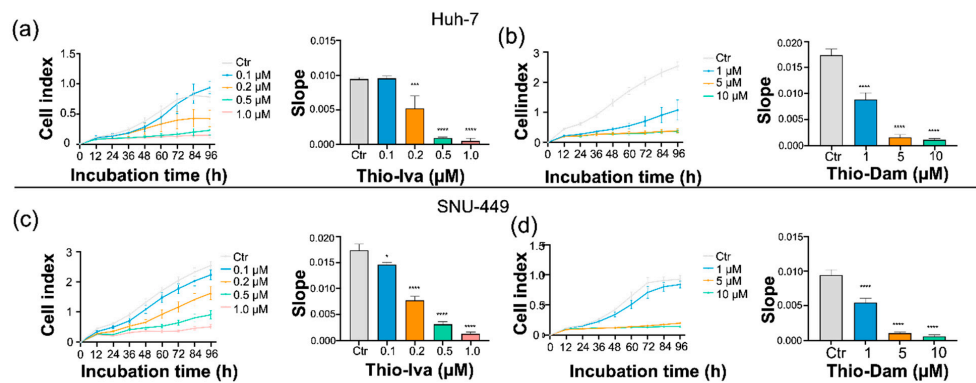


Figure 3. Real-time proliferation detection by iCELLigence. Dose-dependent effects of Thio-Iva and Thio-Dam on the cell index (CI) and its slope over time in Huh-7 (a,b) and SNU-449 cells (c,d). Statistical significance * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$ by ordinary one-way ANOVA as compared to untreated control. All results were expressed as means \pm SEM of 3 independent experiments.

Calculating the slope of the cell index that reflected the proliferation dynamics over time revealed a dose-dependent and highly significant decline to almost zero which came after incubation with the rising concentrations of Thio-Iva and Thio-Dam, both in Huh-7 (Figure 3b) as well as in SNU-449 (Figure 3d) cells, thus impressively showing the antiproliferative effect of both compounds. Furthermore, the CI graphs also revealed that the onset of Thio-Iva-induced growth inhibition occurred after ~ 24 h, while that of Thio-Dam had already occurred after only ~ 12 h.

In line with the iCELLigence proliferation data, long-term surveys (14 days) employing clonogenic assays also yielded a highly significant and dose-dependent reduction in the colony formation of Huh-7 (Figure 4a,b) and SNU-449 (Figure 4c,d) cells by $>90\%$ after Thio-Iva (0.1–0.4 μM) and Thio-Dam (0.5–5 μM) treatment, respectively. In both cell models, the anti-clonogenic effects of Thio-Iva and Thio-Dam exceeded by far the effect of the clinically relevant kinase inhibitor sorafenib (10 μM) (Figure 4b,d).

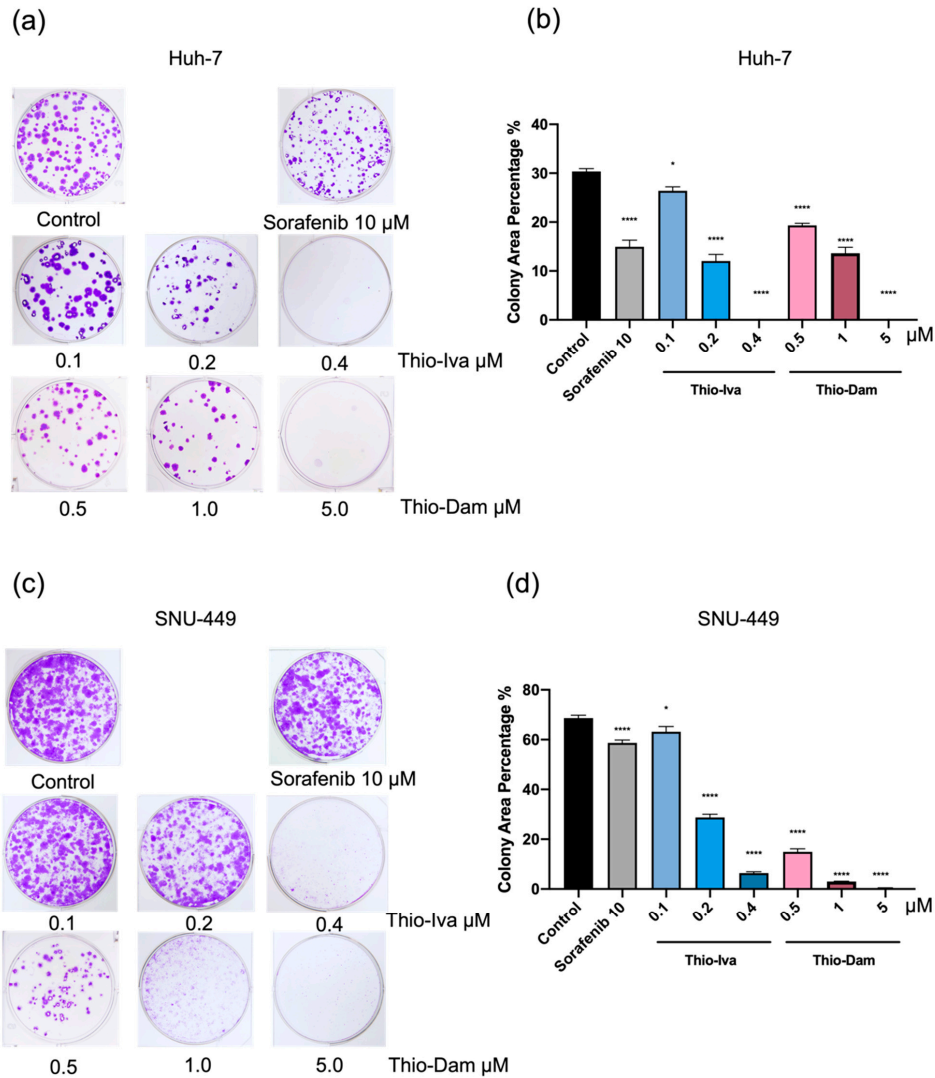


Figure 4. Clonogenic growth. Treatment of Huh-7 (a,b) and SNU-449 cells (c,d) with Thio-Iva, Thio-Dam, and sorafenib. The percentage of area occupied by the colonies was quantified using ImageJ software 14 days after plating. * $p < 0.05$ and **** $p < 0.0001$ by ordinary one-way ANOVA compared to control (untreated). All results were expressed as means \pm SEM of ≥ 3 independent experiments.

3.1.4. Unspecific Cytotoxicity

Unspecific cytotoxicity was evaluated by measuring LDH release into the supernatant of the Huh-7 (Figure 5a) and SNU-449 (Figure 5b) cell cultures after incubation for 6 and 24 h with Thio-Iva and Thio-Dam (1 and 10 μM), respectively. An increase of LDH levels in the supernatant indicates the nonspecific damage of cell membranes, which are not permeable to LDH in their undamaged state. However, even upon treatment with a high concentration of 10 μM , neither Thio-Iva nor Thio-Dam induced statistically significant increases in cytotoxicity after 6 h or 24 h, indicating that both compounds do not affect cell

membrane integrity. Thus, an induction of immediate cytotoxicity is unlikely to account for the observed antiproliferative effects of the novel inhibitors.

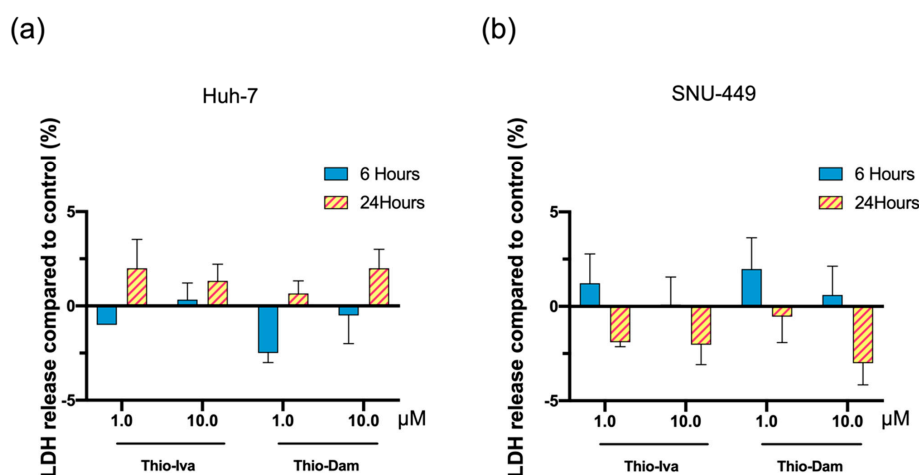


Figure 5. Cytotoxic effects of Thio-Iva and Thio-Dam. Release of lactate dehydrogenase (LDH) after incubation of Huh-7 (a) and SNU-449 (b) cells with 1 and 10 μM of Thio-Iva and Thio-Dam for 6 and 24 h, respectively. LDH release was not significantly altered when compared to untreated controls (set to 0%), indicating that unspecific toxicity did not contribute to the observed effects. Means ± SD of $n = 3$ independent experiments.

3.1.5. Apoptosis Induction and Regulation in HCC Cells

Apoptosis is the most prominent form of programmed cell death, which is mediated by the activation of effector caspases-3, -6, and -7. As procaspases, these proteases are usually inactive in non-malignant cells, which, however, can undergo autolytic activation upon stimulation to form active caspases. Among these caspases, caspase-3 is responsible for most proteolytic processes during apoptosis. Therefore, the detection of activated caspase-3 is a common marker for apoptosis [25].

Treatment with Thio-Iva and Thio-Dam led to a significant and dose-dependent increase in caspase-3 activity in Huh-7 and SNU-449 cells, which was even stronger than those of sorafenib (10 μM). Thio-Iva and Thio-Dam showed significant dose- and time-dependent increases in caspase-3 activity. At 10 μM, Thio-Iva led to an approximately 3-fold increase when compared with untreated cells, and a 5.5-fold increase after 48 h. For SNU-449, a 3-fold increase was also seen after 24 h, and even a 5.5-fold increase after 48 h. Analogously, Thio-Dam (10 μM) showed a 3.5-fold increase in Huh-7 after 24 h, and a 5-fold increase after 48 h, but only a 2-fold increase was observed for SNU-449 after 48 h (Figure 6a,b).

However, both of the thiophene derivatives induced a more pronounced caspase-3 activation in Huh-7 and SNU-449 cells than sorafenib (10 μM). Western blot analyses revealed that Thio-Iva induced apoptosis so as to promote poly-(ADP-ribose)-polymerase (PARP) cleavage in treated HCC cells (Figure 6c), while the effect of Thio-Dam was less pronounced in SNU-449 cells or was even absent in Huh-7 cells.

Increased formation of reactive oxygen species (ROS) is a cell damage mechanism that plays an important role in cancer development and is also known as a trigger of mitochondria-driven apoptosis. The ROS-specific dye CellROX orange was applied to detect Thio-Iva and Thio-Dam-induced ROS formation in the cytoplasm of Huh-7 cells. After incubation for 24 h, a dose-dependent induction of ROS formation was observed in the cytoplasm of treated cells (Figure 6d).

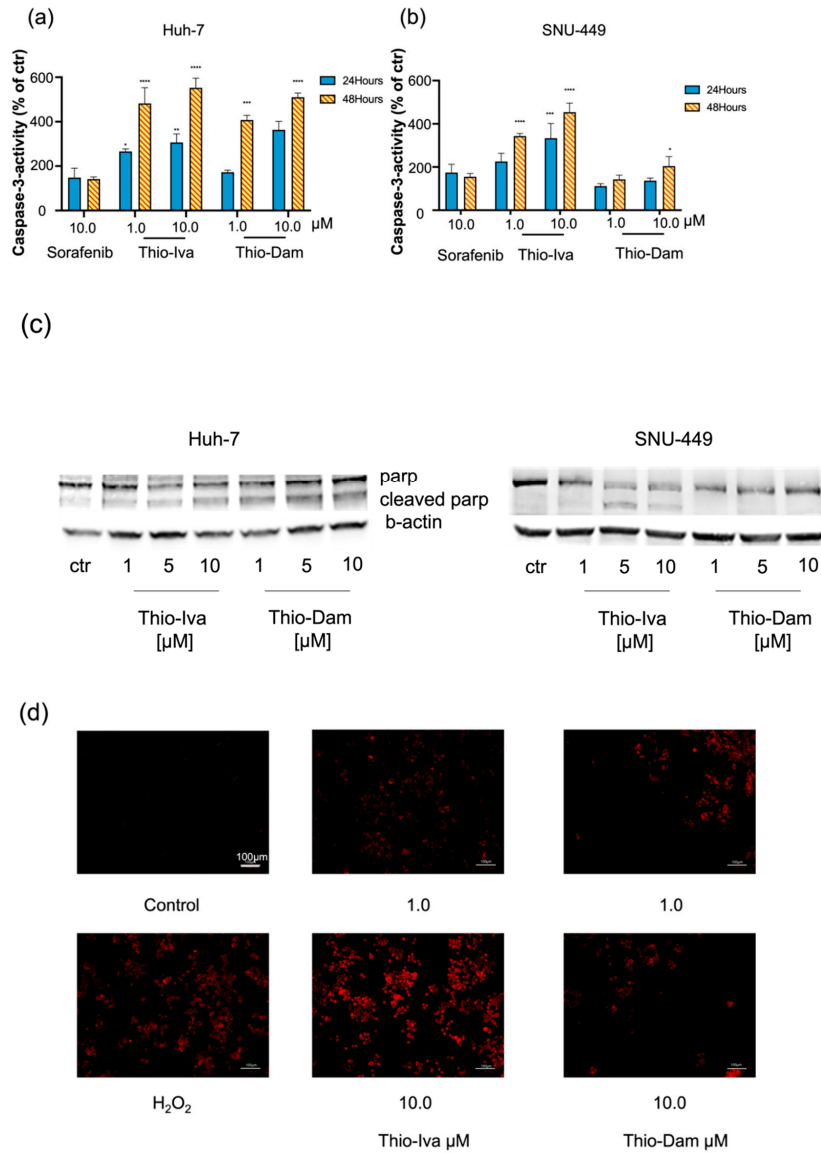


Figure 6. Induction of Apoptosis. (a) Dose- and time-dependent caspase-3 induction in Huh-7 cells after treatment with 1 and 10 μM of Thio-Iva and Thio-Dam and 10 μM sorafenib for 24 h and 48 h. (b) Dose- and time-dependent caspase-3 induction in SNU-449 cells after treatment with 1 and 10 μM of Thio-Iva and Thio-Dam and 10 μM sorafenib for 24 h and 48 h. Results are given as means ± SEM of $n = 3$ independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by ordinary one-way ANOVA compared to untreated controls. (c) Representative Western blot results out of $n = 3$ independent experiments, showing PARP and cleaved PARP expression change by treatment, induced in Huh-7 and SNU-449 cells after 48 h. β-actin was used as loading control. (d) Detection of ROS induction by Thio-Iva and Thio-Dam in HCC cells after 24 h. H₂O₂ served as a positive control. Scale bar, 100 μm.

3.1.6. Cell-Cycle Regulation

The impact of Thio-Iva and Thio-Dam on the cell cycle of Huh-7 and SNU449 cells was determined by flow cytometry. Cells that were treated with 1, 5, and 10 μM of Thio-Iva and Thio-Dam for 48 h showed a dose-dependent and pronounced arrest in the G2/M phase of the cell cycle and a concomitant decrease of cells in the G0/1- and S-phases (Figure 7a–d). By contrast, sorafenib (10 μM) failed to induce a pronounced cell cycle arrest in Huh-7 or SNU-449 cells.

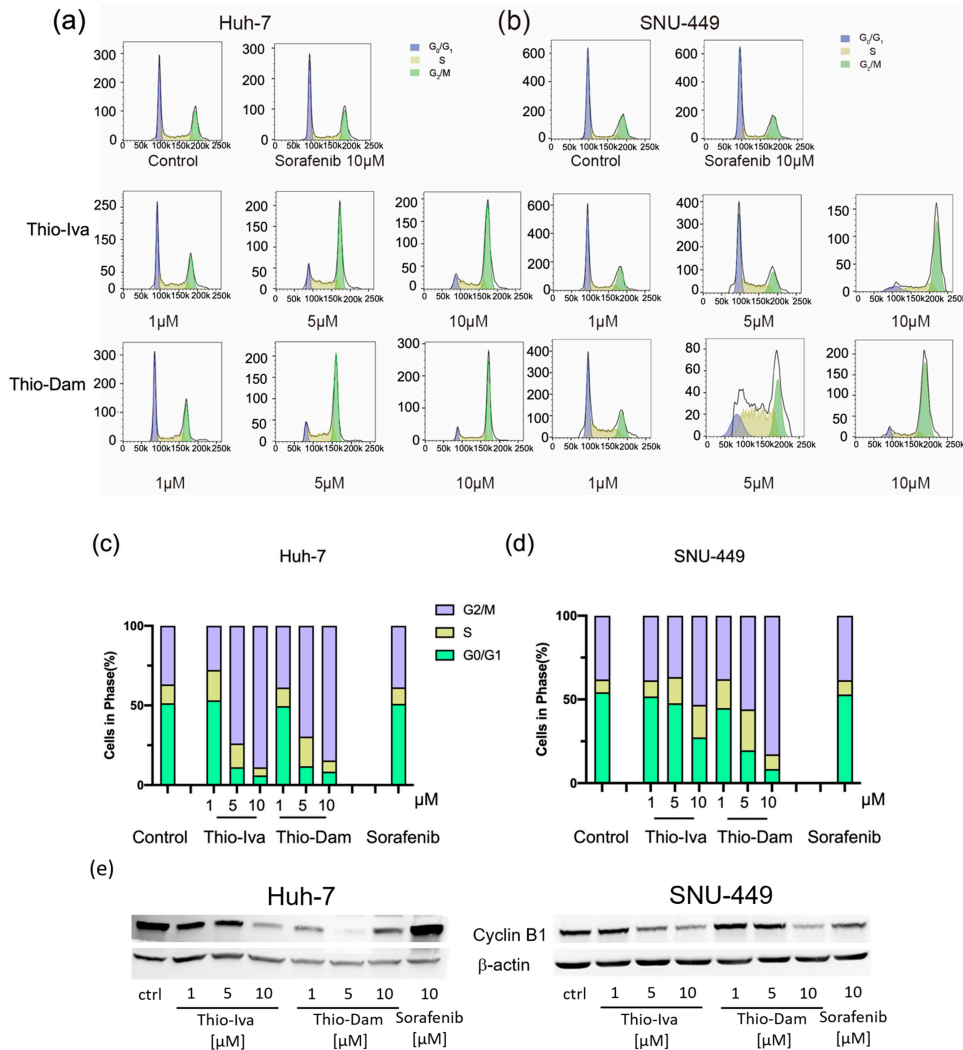


Figure 7. Flow cytometry revealed that Thio-Iva and Thio-Dam induced a pronounced G₂/M arrest after 48 h in Huh-7 (a) and SNU-449 (b) cells. Quantification of the rate of the entire cell cycle; histogram shows average results for Huh-7 (c) and SNU-449 (d). All results are expressed as means \pm SEM of $n = 3$ independent experiments. (e) Representative Western blots of $n = 3$ independent experiments showing cyclin B1 expression change in Huh-7 and SNU-449 cells upon treatment for 48 h. β -actin was used as loading control.

Cyclin B1, a key component in the control of cell cycle progression from the G2 to the M phase, has been implicated in the tumorigenesis and the development of malignancy. Cells suppress and degrade the cell cycle promoter, cyclin B1, in order to escape mitosis [28]. The expression of cyclin B1 was determined to further decipher the molecular mechanism of the G2/M phase blockade by Thio-Iva and Thio-Dam. After 48 h of incubation, both Thio-Iva and Thio-Dam down-regulated cyclin B1 in a dose-dependent manner (Figure 7e), thereby fitting to the flow cytometry findings on a G2/M arrest by the thiophene derivatives in the Huh-7 and SNU-449 cells. The lack of sorafenib to suppress cyclin B1 expression (Figure 7e) corroborates the observation that sorafenib did not induce an appreciable G2/M arrest in either HCC cell line (Figure 7c,d).

3.1.7. Inhibition of Cell Migration

It is mandatory to block the migration and spreading of tumor cells in order to prevent the formation of metastases. Thus, new compounds which inhibit tumor cell migration are of particular interest for the development of new anticancer agents. Wound healing (scratch) assays were performed to investigate the motility of HCC cells treated with Thio-Iva and Thio-Dam (Figure 8). In order to ensure that the scratched gap is filled by migration and not by proliferation, cells were cultured in FBS-free medium for 24 h. The migration rate of untreated Huh-7 control cells was ca. 52.3% after 24 h, while Huh-7 cells treated with Thio-Iva (1, 5, and 10 μ M) acted in a dose-dependent way and showed reduced Huh-7 cell migration rates of 33.0%, 19.0%, and 10.0%, respectively. Thio-Dam (1, 5, and 10 μ M) showed migration rates of 28.0%, 21.3%, and 16.0%, respectively, which are similar to the rates of Thio-Iva (Figure 8a,c). In SNU-449 cells, the migration rate of untreated control cells was 49.3%, and upon Thio-Iva treatment (1, 5, 10 μ M) dropped dose-dependently to 18.3%, 4.6%, and 2.3%, respectively. Comparable results were found for Thio-Dam treatment (1, 5, 10 μ M), which resulted in a drop in SNU-449 migration rates of 8.7%, 2.0%, and 1.7%, respectively (Figure 8b,d).

3.1.8. Antiangiogenic Effects of Thio-Iva and Thio-Dam In Vitro and In Vivo

The effects of Thio-Iva and Thio-Dam on angiogenesis were investigated both in vitro and in vivo. Initially, in vitro tube formation assays with endothelial EA.hy926 cells were performed (Figure 9a). Treatment with Thio-Iva led to a strong inhibition of tube formation, with 51% at 0.2 μ M and even up to 92% at 1 μ M. The effect of Thio-Dam was less pronounced. However, at a dose of 1 μ M, Thio-Dam induced an almost 70% inhibition of tube formation (Figure 9b).

In addition, chicken CAM assays were employed to determine the in vivo anti-angiogenic effects of Thio-Iva and Thio-Dam. Both compounds showed considerable reductions of angiogenesis in a dose-dependent way, which were stronger than those of sorafenib, an established anti-angiogenic drug for the treatment of HCC. The vessels displayed morphological irregularities in response to the treatment with Thio-Iva and Thio-Dam, which were not observed in the untreated controls (Figure 9c,d).

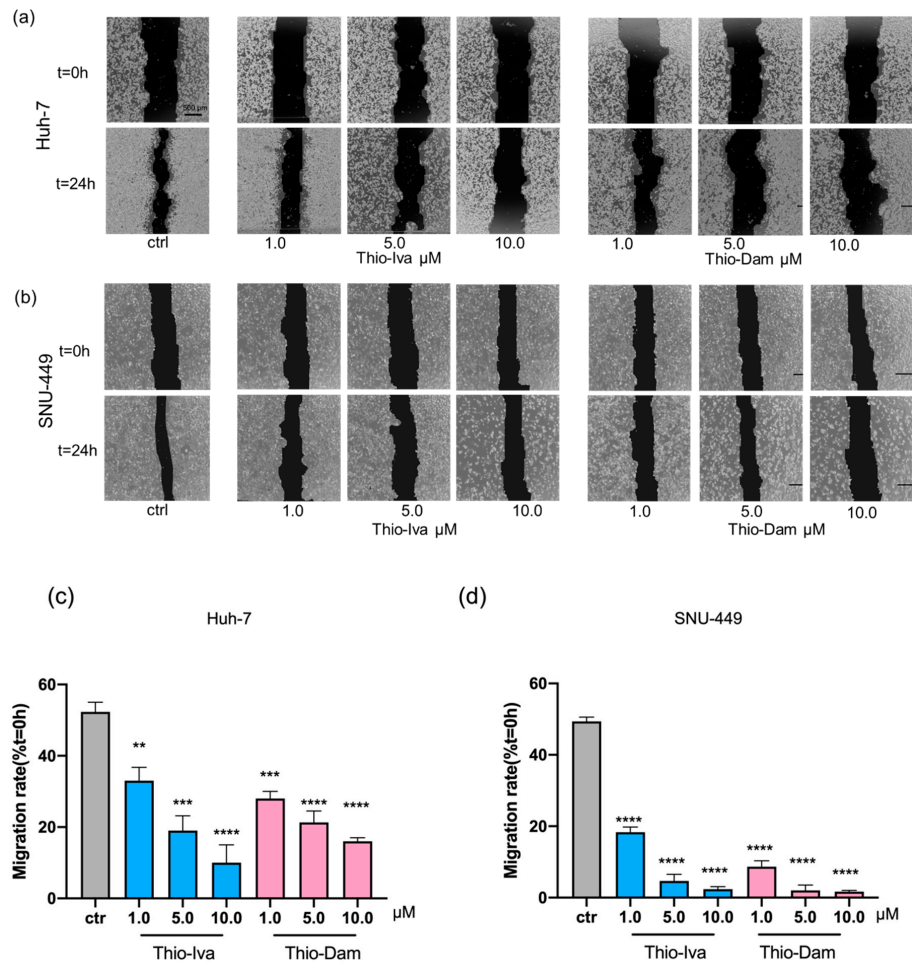


Figure 8. Antimigratory effects of Thio-Iva and Thio-Dam in Huh-7 and SNU-449. (a) Representative images of antimigratory effects of Thio-Iva and Thio-Dam (1–10 μ M) in Huh-7 cells after 24 h. (b) Representative images of antimigratory effects of Thio-Iva and Thio-Dam in SNU-449 cells after 24 h. (c,d) Quantification of the migration rate (in %) of SNU-449 cells after incubation with Thio-Iva and Thio-Dam. ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by ordinary one-way ANOVA compared to untreated controls. Results are given as means \pm SEM of = 3 independent experiments. Scale bar, 500 μ m.

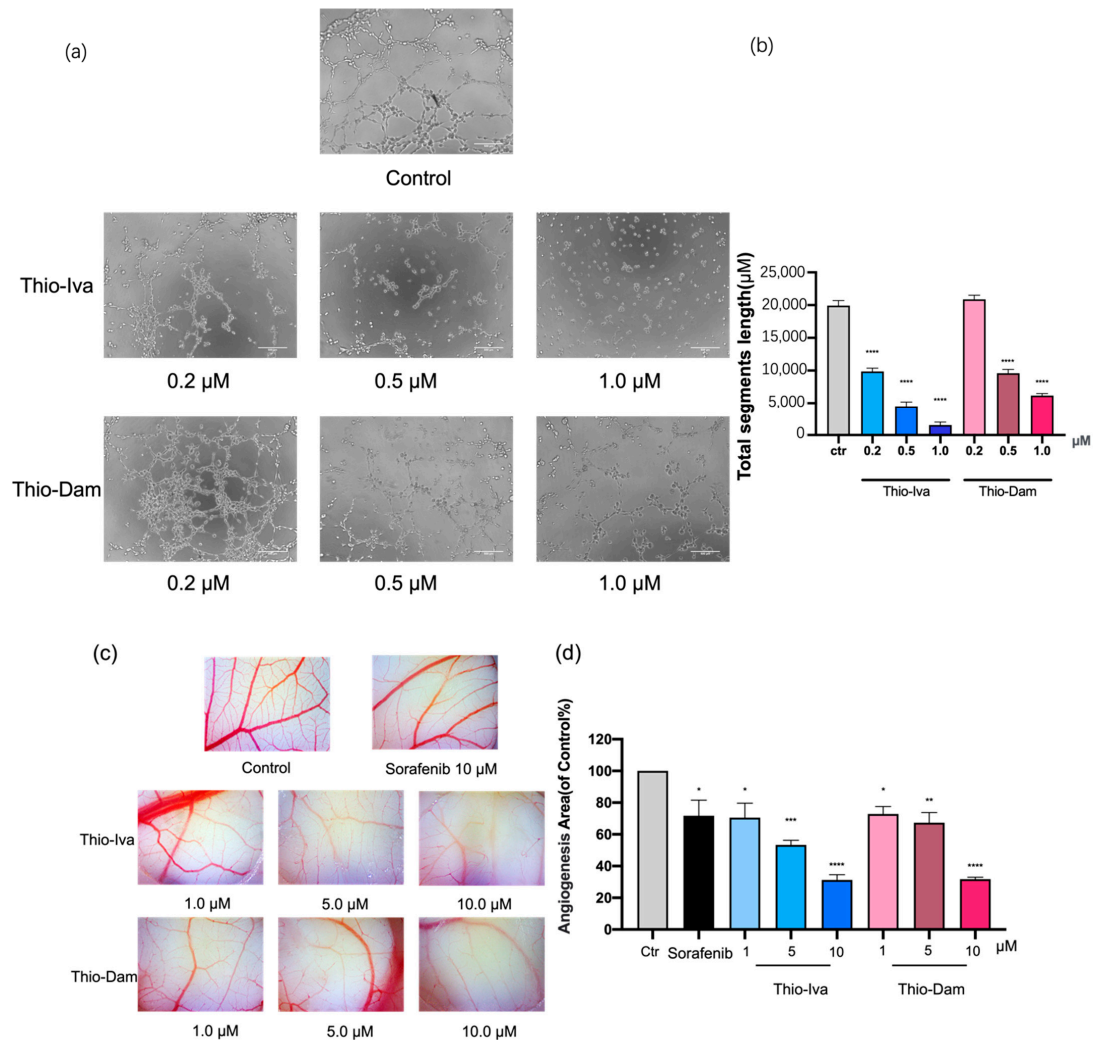


Figure 9. In vitro and in vivo effects of Thio-Iva and Thio-Dam on angiogenesis. (a) Representative images of tube formation assay with EA.hy926 cells. Thio-Iva and Thio-Dam (0.2–1 μ M) were applied for 6 h. (b) Tube formation of EA.hy926 cells was quantified and depicted as changes in total segment length using ImageJ software. (c) CAM assay showing inhibition of angiogenesis in vivo: Representative examples of CAM were taken from a typical experiment. Untreated control is the area outside the silica ring. Inside the silicone ring, the surface was treated with different concentrations of Thio-Iva and Thio-Dam for 48 h. (d) Image-Pro Plus software was used in analysis for blood vessel area quantification (compared to control in %). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by ordinary one-way ANOVA compared to control (untreated area). Results are given as means \pm SEM of = 3 independent experiments.

3.1.9. Antineoplastic Effects of Thio-Iva and Thio-Dam In Vivo

CAM assays were performed to demonstrate the effects of Thio-Iva and Thio-Dam on HCC tumor growth in vivo. Micro tumors of Huh-7 cells were grown on the CAM and were treated with Thio-Iva (1–10 μ M) and Thio-Dam (1–10 μ M) for 72 h. A dose-dependent and highly significant reduction of tumor growth was observed, and the tumor weight decreased by up to 62% (Thio-Iva) and 71% (Thio-Dam), respectively, as compared to PBS-treated controls (Figure 10a,b). Interestingly, sorafenib did not induce a significant reduction of Huh7 microtumor growth in these experiments. It is noteworthy that no increased embryonic lethality rate or signs of developmental retardation was observed in the treated eggs, indicating the good tolerability of the novel compounds—a finding that corroborates the absence of unspecific cytotoxic effects of Thio-Iva and Thio-Dam in Huh-7 and SNU-449 cells in the respective LDH-release assays (Figure 5).

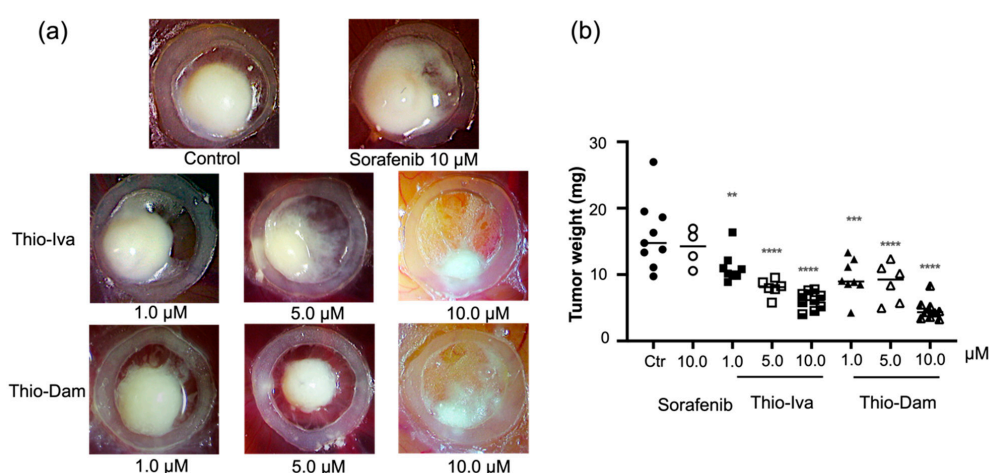


Figure 10. In vivo antineoplastic effects of Thio-Iva and Thio-Dam on Huh-7-derived HCC microtumors. (a) Representative images of Thio-Iva and Thio-Dam-treated microtumors grown on the CAM of fertilized chicken eggs after 72 h. Sorafenib was additionally applied as a clinically relevant HCC therapeutic. PBS-treated microtumors served as controls. (b) Tumor weight analysis. ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ by ordinary one-way ANOVA compared to controls. Results are shown as means \pm SEM of = 3 independent experiments.

4. Discussion

Hepatocellular carcinoma is the fourth leading cause of cancer death in the world, which accounts for more than 80% of global primary liver cancers [29]. In recent years, significant progress has been made in terms of surgical treatment, interventional therapy, and radiotherapy for patients suffering from early HCC [3,30]. However, medical treatment methods for patients with advanced HCC showed only marginal improvements in efficacy [31]. Therefore, there is an urgent need for new drugs for the treatment of advanced HCC. Recently, we introduced a series of 2-(thien-2-yl)-acrylonitriles with different aryl substituents (such as hydroxyl and alkoxy, dialkylamine, and halogen) as protein kinase inhibitory compounds with antineoplastic potency [21].

In the present study, the anti-tumor effects of Thio-Iva and Thio-Dam, the two most effective compounds of the series, were further elaborated in terms of their anticancer properties in two HCC cell models. Their underlying modes of action were deciphered as well as their antineoplastic and anti-angiogenic effects in vivo.

Aggressive tumors are characterized by a sustained proliferation of tumor cells. We showed that the compounds Thio-Iva and Thio-Dam exert strong antiproliferative effects

on HCC cells in a dose- and time-dependent manner, which even exceeded those of the clinically approved multi-kinase inhibitor sorafenib. In addition, real-time proliferation monitoring revealed that Thio-Iva and Thio-Dam exerted their growth inhibitory effects after only 12 and 24 h, respectively. In addition, colony formation assays showed the long-term anti-proliferative effects of the novel thiophene derivatives, while the anticancer effects of Thio-Iva and Thio-Dam were not based on the induction of unspecific cytotoxicity but involved the induction of apoptosis and cell cycle arrest as specific modes of action.

As the evasion of apoptosis by cancer cells is one of the leading causes of uncontrolled tumor cell growth, the acquisition of anti-apoptotic features during carcinogenesis is regarded as one of the hallmarks of cancer [32]. The cysteine protease caspase-3 is the most important executioner caspase of cellular apoptosis [33], and thus its induction by Thio-Iva and Thio-Dam was determined as an unequivocal sign of apoptosis induction in Huh-7 and SNU-449 cells. Treatment with Thio-Iva and Thio-Dam in a time- and dose-dependent manner induced caspase-3 activity, which even exceeded by far those of sorafenib. The increase of ROS levels can promote the dissipation of mitochondrial membrane potential, can cause organ dysfunction, and can trigger mitochondria-driven apoptosis. Moreover, excessive ROS levels are also related to DNA damage [34]. Thio-Iva and Thio-Dam were shown to induce pronounced increases in cytoplasmic ROS levels. Mitochondria are the main source of ROS in cells and the most severely affected organelles of cellular stress [35]. In order to link the Thio-Iva and Thio-Dam-induced rise in cytoplasmic ROS to the mitochondria-driven apoptosis of HCC cells, we demonstrated in this study that the acute burst of ROS in mitochondria specifically causes cell apoptosis and subsequently activates caspase-3. Western blotting showed that PARP, the substrate of caspase-3, was reduced and cleaved into an N-terminal 89 kDa fragment in the Huh-7 cell line incubated with Thio-Iva. Albeit less pronounced, Thio-Dam treatment also decreased PARP expression, at least in SNU-449 cells.

In terms of DNA damage, blocking cell-cycle checkpoints can lead to genome instability and subsequent cell death. G2 abolition prevents cancer cells from repairing DNA damage, forcing them to enter the M phase and the so-called “mitotic catastrophe” as well as apoptosis [36,37]. The G2 checkpoint has become an attractive therapeutic target for anticancer therapy. Flow cytometry analyses revealed a dose-dependent G2/M phase arrest in Huh-7 and SNU-449 cells treated with Thio-Iva and Thio-Dam. These compounds meet the criteria of ideal G2 checkpoint inhibitors, which selectively target molecules that do not participate in the G1 checkpoint or S phase checkpoints [38]. The precise regulation of cyclin B1 is essential for the onset of mitosis and for checkpoint control. It was also shown to regulate cell cycle transition from the G2 to the M phase [39,40]. Thio-Iva and Thio-Dam significantly suppressed cyclin B1 in both cell lines after 48 h, while treatment with sorafenib only had slight effects on cyclin B1 expression, which is in line with our findings from the flow cytometry experiments. More and more evidence suggest that cyclin B1 is highly expressed in several tumors, and its effects were correlated with tumor proliferation, invasion, and apoptosis [40,41]. The distinct suppression of cyclin B1 by Thio-Iva and Thio-Dam can explain the pronounced pro-apoptotic activities of these compounds.

As a malignant, hyper-vascularized solid tumor, HCC can be treated by inhibiting angiogenesis. Tumor angiogenesis is regarded as another hallmark of cancer [32] and may thus be an important target in HCC treatment. Sorafenib inhibits multiple receptor tyrosine kinases such as the VEGFR and PDGFR signaling pathways and has been the first-line drug in the treatment of advanced HCC for a long time [2]. However, a considerable number of HCC patients had to stop sorafenib treatment due to unbearable side effects or drug resistance. Therefore, some other multi-kinase inhibitors such as brivanib, sunitinib, and linifanib were studied, but these failed in phase III trials [11,42,43]. VEGFR-2 is a trans-membrane receptor tyrosine kinase that functions in both physiological and pathological angiogenesis. The activation of VEGFR-2 promotes endothelial cell invasion, migration, proliferation, and angiogenesis [44,45]. However, the VEGFR-2 inhibitor ramucirumab also failed to reach the end point in a recent phase III trial [10]. Thio-Iva and Thio-Dam

were recently described as novel multi-kinase inhibitors with preferential VEGF Receptor inhibition [21]. Endothelial cells are involved in angiogenesis and proliferate to provide the cells required to form new blood vessels. After proliferation, endothelial cells reorganize into a three-dimensional tubular structure [46]. Our in vitro experiments demonstrated that Thio-Iva and Thio-Dam affect EA.hy926 cell tube formation even at low concentrations. In particular, cells treated with Thio-Iva displayed no tubular structure at all. In addition, CAM assays showed that Thio-Iva and Thio-Dam also reduced angiogenesis in vivo and to a higher degree than sorafenib. Vessels in treated CAMs displayed visible morphological irregularities (Figure 7b).

As a prerequisite for invasion and metastasis, the migration of tumor cells is regarded as another hallmark of cancer [32]. HCC cells treated with Thio-Iva and Thio-Dam showed significantly decreased cell migration, indicating that both compounds exert anti-metastatic properties.

The CAM assay is an established model for testing anti-tumor compounds in vivo and can be used as a template for growing micro tumors from human cancer cell lines [47]. We showed that Thio-Iva and Thio-Dam reduce the growth of HCC tumors grown on CAMs, thus confirming their considerable antineoplastic effects in vivo. Both compounds were well-tolerated and did not exhibit embryo toxicity or developmental delay. The effects of the novel thiophene-based compounds were also notably more effective than the treatment with the clinically established multi-kinase inhibitor sorafenib.

5. Conclusions

In conclusion, we demonstrated the pronounced antiproliferative, apoptosis-inducing, antimigratory, and cell cycle-arresting properties of the two novel 2-(thien-2-yl)-acrylonitrile kinase inhibitors in HCC cells. In addition, Thio-Iva and Thio-Dam showed significant antitumor and antiangiogenic effects as well as excellent in vivo tolerance. The novel compounds were shown to effectively attack HCC cells in cellular processes and features that are acquired during carcinogenesis and which are referred to as hallmarks of cancer. Our results show that Thio-Iva and Thio-Dam may provide new and valuable options for the treatment of hepatocellular carcinoma in the future. Hence, future investigations of these promising kinase inhibitors are warranted.

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Lebenslauf

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

Komplette Publikationsliste

1. Schaller, E., A. Ma, L.C. Gosch, A. Klefenz, D. Schaller, N. Goehringer, L. Kaps, D. Schuppan, A. Volkamer, R. Schobert, B. Biersack, B. Nitzsche, and M. Höpfner, *New 3-Aryl-2-(2-thienyl)acrylonitriles with High Activity Against Hepatoma Cells*. *Int J Mol Sci*, 2021. **22**(5).
Impact-Faktor: 6.208
2. Goehringer, N., B. Biersack, Y. Peng, R. Schobert, M. Herling, A. Ma, B. Nitzsche, and M. Höpfner, *Anticancer Activity and Mechanisms of Action of New Chimeric EGFR/HDAC-Inhibitors*. *Int J Mol Sci*, 2021. **22**(16).
Impact-Faktor: 6.208
3. Goehringer, N., Y. Peng, B. Nitzsche, H. Biermann, R. Pradhan, R. Schobert, M. Herling, M. Höpfner, and B. Biersack, *Improved Anticancer Activities of a New Pentafluorothio-Substituted Vorinostat-Type Histone Deacetylase Inhibitor*. *Pharmaceuticals (Basel)*, 2021. **14**(12).
Impact-Faktor: 5.215
4. Ma, A., B. Biersack, N. Goehringer, B. Nitzsche, and M. Höpfner, *Novel Thienyl-Based Tyrosine Kinase Inhibitors for the Treatment of Hepatocellular Carcinoma*. *J Pers Med*, 2022. **12**(5).
Impact-Faktor: 3.508

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