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CONCISE ARTICLE

Chimerically designed HDAC- and tyrosine kinase inhibitors. A series of erlotinib hybrids as dual-selective inhibitors of EGFR, HER2 and histone deacetylases†

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The regulation of chromatin structure and, therefore, transcriptional activity of histone proteins by reversible lysine acetylation is an important posttranslational modification. Inhibitors of histone deacetylase (HDAC) are considered as promising new anti-neoplastic drugs. The hydroxamic acid SAHA *e.g.* is currently used in the treatment of advanced primary cutaneous T-cell lymphoma. The EGFR protein tyrosine kinase inhibitor erlotinib is a prominent drug in cancer chemotherapy and currently approved for treatment of non-small cell lung cancer. In this report, we present a novel strategy for cancer drug development by a combination of EGFR/HER2 kinase and HDAC inhibitory activity in one molecule. By combining two distinct pharmacological properties in one molecule, we expect a broader activity spectrum and less likelihood of drug resistance in cancer patients. The combination led to substances with both HDAC inhibitory properties and EGFR as well as HER2 kinase inhibitory activities.

Introduction

Histone deacetylases (HDACs) are enzymes that remove acetyl groups from an *N*-acetyl lysine amino acid on a histone, thus facilitating high-affinity binding between the histones and DNA backbone and consequently suppressing transcription of the condensed DNA. HDAC proteins are divided into four different subtypes. The first two classes are considered "classical" HDACs, metalloenzymes containing a catalytic zinc atom.[1]

The inhibition of HDACs leads to reversal of tumor suppressor gene silencing, cell cycle arrest, differentiation, and/or apoptosis *via* two mechanisms of action.[2] In brief, the prevention of histone deacetylation maintains an open chromatin structure which facilitates transcription *e.g.* of growth inhibitory or tumor suppressive genes. Moreover, deacetylation of non-histone proteins such as HSP90 by HDAC6 disrupts its association with many oncogenic client proteins like Akt, Raf or the BCR-Abl fusion protein. Induction of ubiquitinylating enzymes such as Ubc8 allows the proteasomal degradation of oncoproteins.[3] HDAC inhibitors (HDACis) also modulate cellular signaling. For example, STAT1 activity is regulated by a balance between phosphorylation and acetylation of this molecule. Increased acetylation of STAT1 mediated by HDACis induces the binding of the tyrosine phosphatase TCP45 which dephosphorylates and inactivates STAT1.[4] Subsequent studies in diffuse large B-cell lymphoma (DLBCL) have shown HDAC3 overexpression and complexation with STAT3 in phospho-STAT3 positive cell lines. Intriguingly, those cell lines were particularly sensitive towards the cytotoxic effects of HDACis.[5] Because of these effects, HDACis have emerged as a new promising drug class for cancer treatment. A number of HDACis have shown promising anti-tumor activity and are now either approved therapeutics or in clinical trials.[6] Most HDACis exhibit a hydroxamic acid or benzamide motif as a Zn complexing head group like belinostat **1** (PXD101, phase III),[7] vorinostat **2** (SAHA, approved for the treatment of advanced primary cutaneous T-cell lymphoma),[6] panobinostat **3** (LBH589, phase III),[8] entinostat **4** (MS-275, phase II)[9] and mocetinostat (MGCD0103, phase I and II) (**5**).

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‡ Due to his untimely death, our highly respected colleague Dr Thomas Beckers can no longer be a corresponding author of the pharmacological part of this publication. R.I.P.

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The HER family of receptor tyrosine kinases represents another heavily exploited target class for solid tumor treatment. Many targeted agents including antibodies (trastuzumab, cetuximab, and panitumumab) or small molecule kinase inhibitors (gefitinib, erlotinib, and lapatinib) addressing either EGFR or HER-2 are nowadays a fundamental part of targeted cancer treatment including non-small cell lung cancer (NSCLC).[10] However, it is not solely the overexpression of a specific receptor tyrosine kinase that is a common denominator of tumor sensitivity towards a particular kinase inhibitor. Despite frequent overexpression in NSCLC, it was found that only a small fraction of patients whose tumors harbor sensitizing mutations in the kinase domain would benefit most from EGFR directed kinase inhibitors.[11] Later on it was found that somatic mutations in *KRAS* play a pivotal role in conferring *de novo* resistance to EGFR kinase inhibitors in NSCLC and are a predictor of poor response of metastatic colorectal cancer patients to EGFR antibodies.[12] Today, a non-mutated *KRAS* status actually is an eligibility criterion for colorectal cancer patient treatment with the EGFR blocking antibody panitumumab. These examples indicate that it is the overall altered molecular pathway pattern rather than a single target dependency that determines tumor aggressiveness on the one hand and drug sensitivity on the other hand. In clinical practice this is reflected by the fact that targeted agents are often accompanied by classical chemotherapy in order to achieve optimal therapeutic results. The therapeutic promise of combining HDACis with inhibitors of tyrosine kinases (TKIs) is further substantiated by a most recent clinical study in which resminostat appeared to resensitize hepatocellular carcinomas towards sorafenib.[13]

To expand this principle towards further HDACi/kinase inhibitor combinations and to improve the therapeutic efficacy of currently approved kinase inhibitors such as the EGFR inhibitor erlotinib **6** it appears obvious to combine kinase inhibitors with a regimen that may disrupt pathways conferring resistance towards targeted cancer treatment. HDACis, through modification of both histone and non-histone substrates, have the potential to disrupt multiple pathways of resistance. We attempted to combine HDAC inhibition and EGFR inhibition in a single molecular entity. A direct modulation of EGFR turnover by HDAC6 has recently been shown.[14]

In principle, inhibition of both molecular mechanisms may be achieved by a simple combination therapy. The possible synergetic effects of a combination therapy of HDAC and EGFR inhibitors for cancer patients are currently investigated.[1] This is however often impeded by other constraints such as different pharmacokinetics, drug–drug interactions, toxicity, bioavailability or simply patient compliance. Our main goal therefore was not to discover molecules optimized for synergistic effects but rather on the development of a single moiety that retains dual mechanism of action.[1,10]

In this report we present a novel strategy for anticancer drug development, by combining the pharmacological activity of EGFR/HER2 kinase inhibition with inhibition of HDAC class I/II enzymes.

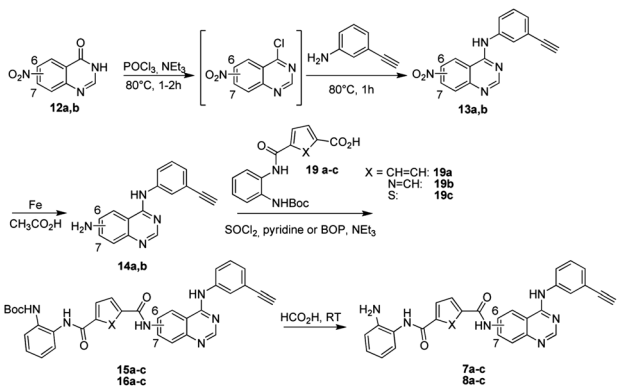
Compound **6** was selected as a scaffold to include a HDAC inhibitory head group ("warhead") in order to combine the antiproliferative activity of EGFR/HER2 inhibition with the proapoptotic, transcriptional reprogramming activity of a HDACi. Of the various known HDAC inhibitory head groups, we chose the hydroxamic acid and benzamide motifs for combination with the *N*-phenylquinazoline-4-amine core structure of **6** to obtain chimerically designed compounds. The feasibility of this approach was previously shown by our group by the combination of the biologically active parts of the tyrosine kinase inhibitors imatinib and lapatinib with HDAC inhibitory head groups.[15,16]

Results and discussion

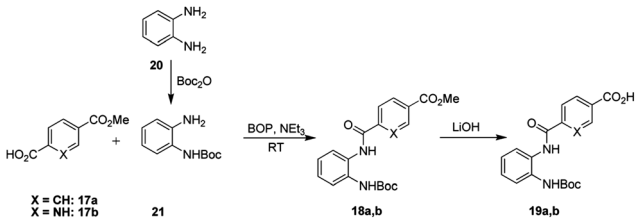
Chemistry

The erlotinib-hybrids containing an amide substructure (**7a–c**, **8a–c**) were synthesized as follows: preparation of 6-nitroquinazoline-4(3*H*)-one (**12a**)[17] and 7-nitroquinazoline-4(3*H*)-one (**12b**)[18] from 2-amino-5-nitrobenzonitrile and from 2-amino-4-nitrobenzoic acid, respectively, as reported[16] was followed by chlorination and subsequent S_NAr-reaction of the resulting intermediate 4-chloronitroquinazoline with 3-ethynylaniline in a one pot synthesis according to *Nishino*.[19] *N*-(3-Ethynylphenyl)-6-nitroquinazoline-4-amine (**13a**) and *N*-(3-ethynylphenyl)-7-nitroquinazoline-4-amine (**13b**) thus obtained were reduced by use of iron in acetic acid as described by Rachid *et al.*[17] to get corresponding 6-aminoquinazoline-4(3*H*)-one (**14a**)[20] and 7-aminoquinazoline-4(3*H*)-one (**14b**),[20] which were used as central intermediates for amidation with the suitably protected and substituted carboxylic acids (**19a–c**).[15] These amidations were either performed by transformation of the corresponding carboxylic acid to its carboxylic acid chloride in a mixture of pyridine–thionyl chloride and addition of the primary arylamino compounds or mediated by BOP [1-benzotriazolyl-oxy-tris-(dimethylamino)phosphonium-hexafluoro-phosphate] as a coupling reagent. Deprotection of the *tert*-butyl-2-benzamido-carbamates with formic acid led to the desired target compounds **7a–c**, **8a–c**.

The substituted carboxylic acids, 4-[2-(*tert*-butoxycarbonylamino)phenylcarbamoyl]benzoic acid (**19a**) and 6-[2-(*tert*-butoxycarbonylamino)phenylcarbamoyl]nicotinic acid (**19b**), which were used for the central amidation step as described in Scheme 1 were prepared from the corresponding methoxy-carbonylaroylic acids (**17a–c**, Scheme 2), by reaction with *tert*-butyl-2-aminophenylcarbamate (**21**)[21] followed by selective alkaline cleavage of the methyl ester with LiOH



Scheme 1



Scheme 2 Preparation of 4-[2-(*tert*-butoxycarbonylamino)phenylcarbamoyl]benzoic acid (**19a**) and 6-[2-(*tert*-butoxycarbonylamino)phenylcarbamoyl]nicotinic acid (**19b**).

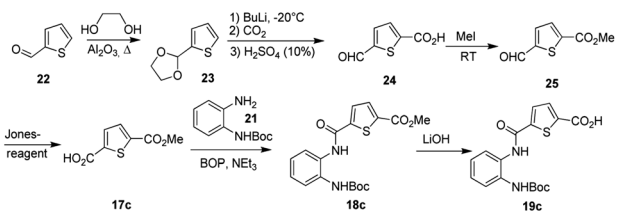
(Scheme 2). The mono-protected phenylenediamine **21** was easily accessible by reaction of *o*-phenylenediamine (**20**) with Boc_2O in THF solution.[15]

Following the same synthetic route, 5-[2-(*tert*-butoxycarbonylamino)phenylcarbamoyl]thiophene-2-carboxylic acid (**19c**) was prepared from 5-(methoxycarbonyl)thiophene-2-carboxylic acid (**17c**)[22] (Scheme 3). **17c** hereby was obtained from thiophene-2-carbaldehyde (**22**) by transformation to 2-(thiophene-2-yl)-1,3-dioxolane (**23**),[23] lithiation with *n*-BuLi, introduction of the carboxylic acid and cleavage of the 1,3-dioxolane in one step,[23] followed by esterification of the carboxylic acid[22] with methyl iodide.

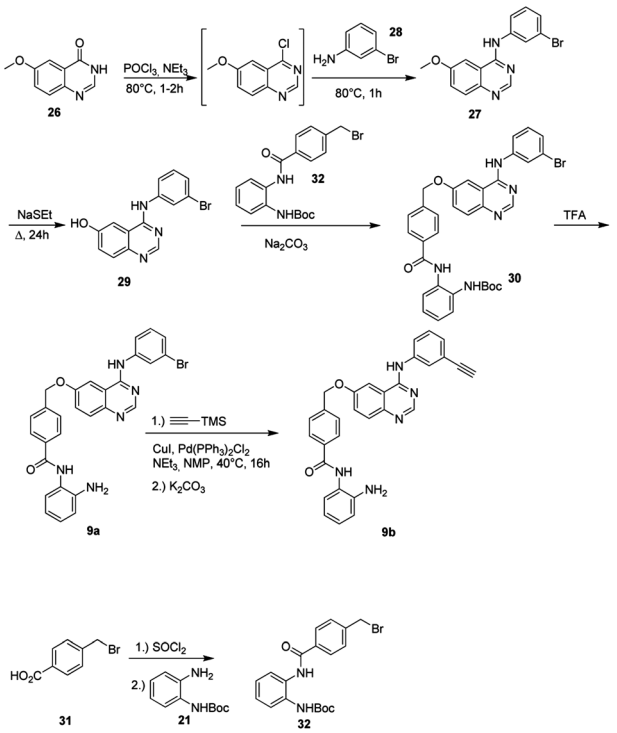
For the synthesis of *N*-(2-aminophenyl)-4-{[4-(3-ethynylphenylamino)quinazolin-6-yloxy]methyl}benzamide (**9b**, Scheme 4), a compound combining the *N*-(3-ethynylphenyl)quinazoline-4-amine substructure of erlotinib **6** and linking the HDAC-head group by an ether increment, the synthetic approach was modified. 6-Methoxyquinazoline-4(3*H*)-one (**26**) was prepared according to Nishino *et al.*[24] from 2-amino-5-methoxybenzoic acid, followed by the one pot synthesis of *N*-(3-bromophenyl)-6-methoxyquinazoline-4-amine (**27**) from 6-methoxyquinazoline-4(3*H*)-one (**26**) and 3-bromoaniline (**28**) analogous to *Nishino*.[19] After treatment with NaSEt, the free phenol **29** was coupled with *tert*-butyl-2-[4-(bromomethyl)-benzamido]phenylcarbamate (**32**). Cleavage of the urethane with TFA led to *N*-(2-aminophenyl)-4-{[4-(3-bromophenylamino)-quinazoline-6-yloxy]methyl}benzamide (**9a**). The desired *N*-(2-aminophenyl)-4-{[4-(3-ethynylphenylamino)quinazoline-6-yl-oxy]methyl}benzamide (**9b**) was prepared by a Pd-catalyzed Sonogashira reaction with TMS-protected acetylene following deprotection with K_2CO_3.

The *tert*-butyl-2-[4-(bromomethyl)benzamido]phenyl-carbamate (**32**) was easily accessible by amidation of 4-(bromomethyl) benzoic acid (**31**), activated with $SOCl_2$, with the mono-protected phenylenediamine **21** (Scheme 4).

The substituted carboxylic acid **38** was synthesized according to Scheme 5. The quinazoline-4(3*H*)-one system **33** (Scheme 5) was prepared according to Lit.[24] from 2-aminobenzoic acid,



Scheme 3 Preparation of 5-[2-(*tert*-butoxycarbonylamino)phenylcarbamoyl]thiophene-2-carboxylic acid (**19c**).

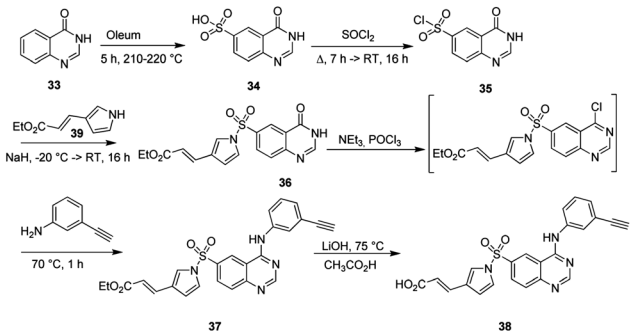


Scheme 4 Synthesis of *N*-(2-aminophenyl)-4-((4-(3-ethynylphenylamino)quinazoline-6-yloxy)methyl)benzamide (**9b**), combining the *N*-(3-ethynylphenyl)quinazoline-4-amine substructure of erlotinib **6** with the HDAC-head group by an ether.

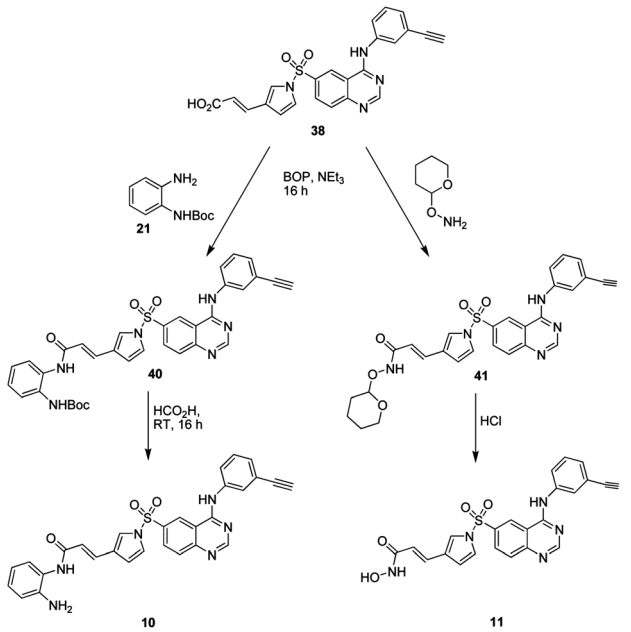
followed by sulfonation with oleum. After activation of the sulfonic acid **34** with $SOCl_2$, the chloride **35** was substituted with (*E*)-ethyl-3-(1*H*-pyrrol-3-yl)acrylate (**39**).[25] Analogous to *Nishino*,[24] the resulting (*E*)-ethyl-3-[1-(4-oxo-3,4-dihydro-quinazoline-6-ylsulfonyl)-1*H*-pyrrol-3-yl]acrylate (**36**) reacted in a one pot synthesis with 3-ethynylaniline to (*E*)-ethyl-3-{1-[4-(3-ethynylphenylamino)quinazoline-6-ylsulfonyl]-1*H*-pyrrol-3-yl}acrylate (**37**).

The carboxylic acid (*E*)-3-{1-[4-(3-ethynylphenylamino)-quinazoline-6-ylsulfonyl]-1*H*-pyrrol-3-yl}-acrylic acid (**38**) was obtained by treatment with LiOH.

The title compounds (**10**, **11**) of Scheme 6 were synthesized by combining (*E*)-3-{1-[4-(3-ethynylphenylamino)quinazoline-6-ylsulfonyl]-1*H*-pyrrol-3-yl}acrylic acid (**38**) with mono-protected



Scheme 5 Synthesis of the intermediate (*E*)-3-{1-[4-(3-ethynylphenylamino)quinazoline-6-ylsulfonyl]-1*H*-pyrrol-3-yl}acrylic acid (**38**).



Scheme 6 Synthesis of (*E*)-*N*-(2-aminophenyl)-3-{1-[4-(3-ethynylphenylamino)quinazoline-6-ylsulfonyl]-1*H*-pyrrol-3-yl}acrylamide (**10**) and (*E*)-3-{1-[4-(3-ethynylphenylamino)quinazoline-6-ylsulfonyl]-1*H*-pyrrol-3-yl}-*N*-hydroxyacrylamide (**11**).

phenylenediamine **21** (ref. 15) or commercially available *O*-(tetrahydro-2*H*-pyran-2-yl)hydroxylamine followed by deprotection.

Biology

The ability of compounds **7–11** to inhibit HDACs was tested in both, biochemical and cellular assays. Biochemical assays relied on recombinant HDAC isoenzymes expressed either in HEK293 cells (HDAC1 and 6) or in SF21 cells (HDAC3 and 8) and were conducted to determine isoenzyme or class selectivity of HDAC inhibition. The underlying biochemical principle is that the release of the fluorophore AMC by HDAC activity from a fluorogenic peptide substrate ((Ac-NH-GGK(Ac)-AMC) is measured in a fluorescence reader.[26] The nuclear extract contains a broad range of different HDAC isoenzymes (HDAC 1–3, 5 and 8) and therefore allows conclusions about the inhibitory activity on various HDAC enzymes. The cellular assays allow conclusions about the activity of compounds **7–11** within cells. Its principle relies on using the cell-permeable fluorogenic substrate Boc-K(Ac)-7-amino-4-methylcoumarin (AMC). After penetration into HeLa cells, the deacetylated product Boc-K-AMC is formed which, after cell lysis, is cleaved by trypsin, finally releasing the fluorophore AMC.

The *in vitro* data for inhibition of HeLa nuclear extract HDAC activity, a mixture of HDAC isoenzymes **1–3**, **5** and **8**, cell HDAC inhibition, recombinant rHDAC 1, rHDAC 3, rHDAC 6 and rHDAC 8 in comparison to SAHA **2** and erlotinib **6** as reference compounds are shown in Table 1.

Combining the structural features of an *N*-(3-ethynylphenyl)-quinazoline-4-amine with an *N*-(2-aminoaryl)benzamide motif by linking the respective amide with the 6-position of the quinazoline led to compounds **7a–c** (Table 1) with no significant inhibition of HDAC and HDAC 6 in nuclear extracts. The compounds **8a–c** employ the same structural motives, but the amide is linked with the quinazoline at the 7-position. On an enzymatic level, these compounds display an effect on nuclear HDAC inhibition in contrast to the respective 6-substituted isomers, albeit the overall inhibition is at 30% max. Compound **9b** contains the *N*-(3-ethynylphenyl)-quinazoline-4-amine substructure of erlotinib **6** linked to the HDAC-head group by a methylene ether increment, leading to inhibition of nuclear extract HDAC and HDAC 1 in the nanomolar range. Exchange of the ethynyl group (**9b**) with bromine (**9a**) does not significantly change the inhibitory activity.

Linking the erlotinib substructure with an *N*-(2-aminophenyl)-3-(1-sulfonyl-1*H*-pyrrol-3-yl)acrylamide results in inhibition of nuclear extract HDAC and HDAC 1 in the micromolar range (compound **10**). The potency of the phenylenediamine inhibitors depends on the nature of the central linking system between the phenylenediamine and the erlotinib scaffold. In this series of HDAC inhibitors the compounds harboring an oxymethyl-benzamide (**9a**, **9b**) or a sulfonylpyrrol-acrylamide (**10**) show an IC_{50} of inhibition of rHDAC 1 below 100 nM. This methylene-benzamide motif is also found in two benzamides in advanced clinical development named entinostat (**4**) and mocetinostat (**5**) (Fig. 1). The same magnitude of potency is seen in the sulfonylpyrrol-acrylamide (**10**).

Changing the phenylenediamine structure motif to a hydroxamic acid leads to compound **11** with an inhibition of nuclear extract HDAC and HDAC 1 in the low nanomolar range. The IC_{50} values show approximately 10 times higher potency towards nuclear extract HDAC and rHDAC 1 than the reference compound SAHA.

Furthermore, a change in the isoform selectivity was observed.

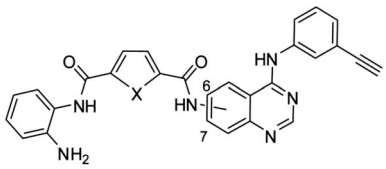
None of the 2-aminophenyl-derivatives displayed any inhibitory activity in the HDAC class II enzyme assays rHDAC 6 or rHDAC 8 under the test conditions. The corresponding hydroxamic acid **11**, however, displayed inhibition of these two isoforms in the sub-μM range. This isoform selectivity is consistent with observations from benzamides and hydroxamates in clinical development.[27]

Since compounds **7–8** did not display the desired HDAC inhibitory activity, they were abandoned from the investigation of the second pharmacological property, namely inhibition of EGFR and Her-2 kinase activity.

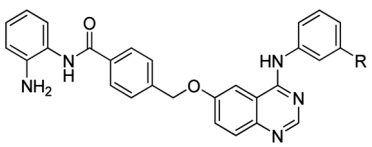
Compounds **9–11** were subsequently subjected to *in vitro* kinase assays that employ recombinant kinases to phosphorylate an artificial poly-Glu-Tyr peptide substrate using radiolabeled ATP. The results are shown in Table 2. Additional tyrosine and serine/threonine kinases from different families were included to investigate whether the spectrum of specificity would be retained.

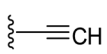
Combination of the erlotinib substructure with an *N*-(2-aminophenyl)-4-(oxymethyl)benzamidyl (**9a**, **b**) or a *N*-(2-aminophenyl)-3-(1-sulfonyl-1*H*-pyrrol-3-yl)acrylamide motif (**10**, **11**) actually conserved tyrosine kinase inhibitory properties, albeit the potencies of **10** and **11** are diminished. Moreover, the selectivity profile towards EGFR/HER1 and HER2 compared to erlotinib did not change significantly. Although inhibition of CDK2 may be more pronounced by **9a**, **9b** and **10**, it is questionable whether any kinase inhibitory activity above an IC_{50} of 10 μM in a biochemical assay is of physiological relevance.

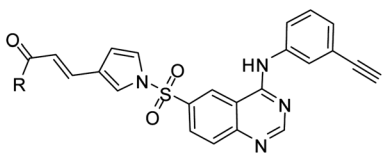
Table 1 Inhibition of nuclear extract HDAC, cell HDAC and rHDAC 1, rHDAC 3, rHDAC 6 and rHDAC 8 (mean values of at least two independent determinations conducted in triplicate)



No.	Position as indicated in the structure	X	Nuclear HDAC inhibition IC_{50} [μM]	Cellular HDAC inhibition IC_{50} [μM]	rHDAC 1 inhibition IC_{50} [μM]	rHDAC 3 inhibition IC_{50} [μM]	rHDAC 6 inhibition IC_{50} [μM]	rHDAC 8 inhibition IC_{50} [μM]
7a	6	CH=CH	>32	>15.8	10.5	0.61	>32	>32
7b	6	N=CH	>32	>15.8	0.31 (70% max)	0.44	>32	>32
7c	6	S	>3	>15.8	8.6	0.92	>32	>32
8a	7	CH=CH	0.2 (30% max)	0.78 (30% max)	0.8	0.5	>32	>32
8b	7	N=CH	0.51 (30% max)	>5.01	5.6	0.32	>32	>32
8c	7	S	0.38 (30% max)	>5.01	2.2 (75% max)	0.77 (70% max)	>32	>32



No.	R	Nuclear HDAC inhibition IC_{50} [μM]	Cellular HDAC inhibition IC_{50} [μM]	rHDAC 1 inhibition IC_{50} [μM]	rHDAC 3 inhibition IC_{50} [μM]	rHDAC 6 inhibition IC_{50} [μM]	rHDAC 8 inhibition IC_{50} [μM]
9a	Br	0.25	2.46	0.074	0.51 (75% max)	>32	>32
9b		0.20 (50% max)	1.85	0.041	0.55	>32	>32



No.	R	Nuclear HDAC inhibition IC_{50} [μM]	Cellular HDAC inhibition IC_{50} [μM]	rHDAC 1 inhibition IC_{50} [μM]	rHDAC 3 inhibition IC_{50} [μM]	rHDAC 6 inhibition IC_{50} [μM]	rHDAC 8 inhibition IC_{50} [μM]
10		7.2 (50% max)	4.24	0.091	0.84 (75% max)	>32	>32
11		0.0064	0.034	0.0065	0.015	0.0073	0.59
SAHA **2**		0.079	0.063	0.013	0.037[a]	0.025[a]	1.2[a]
Erlotinib **6**		>32	>15.8	>32	>32	>32	>32

[a] We published the data for SAHA as reference inhibitor also in ref. 16.

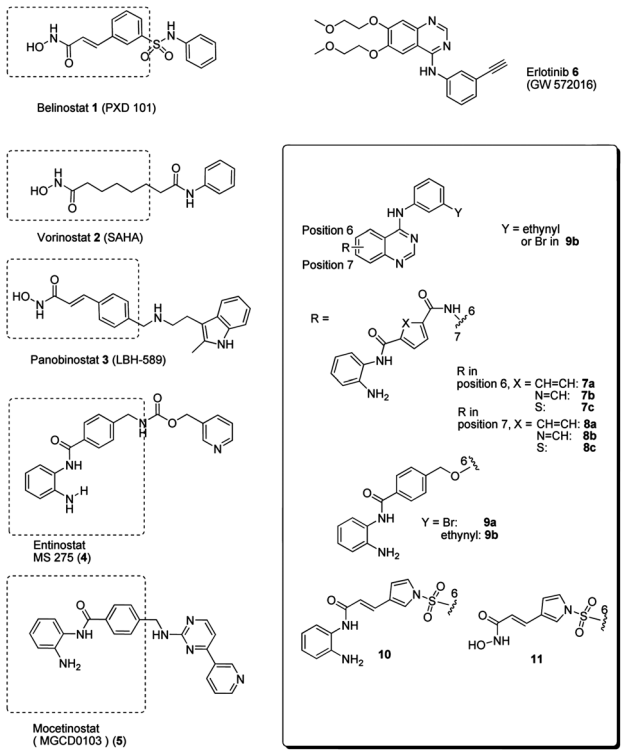


Fig. 1 Chemical structures of selected HDACis (**1–5**) in preclinical and clinical developments, TKI (**6**) and novel chimerically designed compounds (**7–11**).

To complete the pharmacological profiling, we subjected **7–11** including SAHA and erlotinib to cytotoxicity assays using a range of commonly used cancer cell lines, namely HeLa, A549, A431, Cal27 SKBR3 and SKOV3. CAL27 head and neck cancer cells are known to be sensitive towards EGFR kinase inhibitors while SKBR3 mammary carcinoma cells and SKOV3 ovarian carcinoma cells were chosen because of their high expression levels of the HER2. A431 human vulva carcinoma cells overexpress the EGFR and display only marginal amounts of surface HER2. HeLa human cervical cancer cells generally display a good sensitivity towards HDAC inhibitors. A549 human NSCLC cells do not respond to erlotinib, but are sensitive toward HDAC inhibitors. The cytotoxicity data are summarized in Table 3.

The inhibition of HDAC and EGFR/HER-2 by **9a**, **9b**, **10** and **11** is reflected in the cytotoxicity profiles of the compounds, *e.g.* all four compounds show profound activity towards A549 NSCLC cells, which may be attributed to the dual HDAC/EGFR activity. As shown recently, the *KRAS*-mutated NSCLC cell line A549, proved to be resistant to erlotinib, could be sensitized by cotreatment with a HDACi.[28]

In our own investigations the combination of the HDACi SAHA **2** and erlotinib **6** according to Chou–Talalay by using the CalcuSyn software[29] exhibited a significant synergism in cytotoxicity on the cell line A549 as depicted by a combination index value of 0.44 ± 0.11 ($r = 0.99$) at the ED_{50} level.

A particular sensitivity towards CAL27 cells may be due to their kinase inhibitory pharmacological properties. Interestingly **11**, which shows the overall most favourable binary activity (HDAC inhibition and inhibition of EGFR/HER-2), also displays the most potent cytotoxicity. The pronounced cytotoxicity of **8a–c** cannot be explained by inhibition of HDAC activity, thus a different mode of action might be postulated as the underlying cause of cytotoxicity. On the other hand, the lack of cytotoxicity toward SKBR3 and SKOV3 cells argues against a broad unspecific toxicity profile of **8a–c**. The activity of **7a–c** on CAL27 might be attributed to their EGFR inhibitory activity which is 8.3, 39 and 66 nM for compounds **7a–c**, respectively.

Conclusions

The combination of the *N*-(3-ethynylphenyl)quinazoline-4-amine structure of erlotinib **6** with an *N*-(2-aminoaryl)benzamide motif linked by an amide spacer did not result in a significant inhibition of nuclear extract HDAC (compounds **7a–c** and **8a–c**). By combining the two partial structures by a methylene ether as a linker, a selective inhibition of HDAC class I isoforms as well as of nuclear and cell HDAC in the submicromolar range was accomplished (compound **9b**). The exchange of the ethynyl group with bromide did not diminish activity (compound **9a**).

However, to achieve inhibition of cellular or recombinant HDAC class I and class II isoforms in the low nanomolar range, we had to change the benzamide motif towards a hydroxamic acid. The resulting compound **11** showed similar or improved HDAC inhibition in comparison to SAHA (**2**).

Moreover, compound **11** is acting as a true pharmacologically chimeric compound, exhibiting cytotoxic activity on all tested tumor cell lines and displaying activity against EGF and HER2 receptors.

The only benzamide compounds with pharmacologically chimeric properties were **9a** and **9b** with observed cytotoxicity against the tumor cell lines in the low micromolar or high nanomolar range and inhibition of EGF and HER-2 receptors. Future studies will aim at the pharmacological characterization of the HDAC/EGFR/HER-2 kinase inhibitors with regard to their potential to provide additional benefit by displaying two

Table 2 IC_{50} data for the inhibition of EGFR and HER2 kinase activity by compounds **9–11** in μM. Inhibition of PDGF-Rß, InsR, Abl1, CDK2, PKA and Plk1 was included to profile specificity (mean values of at least two independent determinations conducted in triplicate)

No.	EGFR	HER2	PDGF-Rß	InsR	Abl1	CDK2	PKA	Plk1
9a	0.033	0.039	28.00	150.00	11.00	22.00	>100.00	>100.00
9b	0.078	0.066	7.20	36.00	7.00	13.00	>100.00	>100.00
10	1.3	2.9	16.00	41.00	15.00	17.00	24.00	27.00
11	1.2	3.4	70.00	65.00	27.00	>100.00	>100.00	>100.00
Erlotinib **6**	0.005	0.12	2.30	>100.00	2.10	>100.00	>100.00	>100.00

Table 3 IC_{50} data for cytotoxicity on HeLa, A549, A431, Cal27, SKBR3 and SKOV3 in μM (mean values of at least two independent determinations conducted in triplicate)

No.	Position	HeLa	A549	A431	Cal27	SKBR3	SKOV3
7a	6	>50	>50	>16	0.33	>50	>50
7b	6	21.5	39	>16	8.0	11	7.7
7c	6	>50	>50	>16	0.41	>50	>50
8a	7	1.7	1.4	1.6	0.46	39	>50
8b	7	1.5	2.1	0.79	0.45	>50	>50
8c	7	1.8	0.82	0.96	1.2	33	30
9a	Br	1.45	1.3	1.7	0.62	5.1	6.3
9b		1.35	0.9	1.6	0.61	3.4	5.1
10		3.45	3.7	3.7	2.5	27	5.7
11		0.5	0.24	0.26	0.16	0.19	0.56
SAHA **2**		1.5[a]	1.8[a]	4.2[a]	3.2[a]	2.6[a]	2.2[a]
Erlotinib **6**		8.3	>50	2.5	0.074	15	31

[a] Data taken from ref. 16.

mechanisms of action. These studies include assessment of their activity range towards cell lines across broader pathological ranges, cell types with particular desensitizing mutations, potential combination with other targeted therapies and/or classical chemotherapy as well as investigation of their anti-tumor activity compared to SAHA, erlotinib and combinations thereof using human xenograft models.

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