Chimeric tyrosine kinase-HDAC inhibitors as antiproliferative agents

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Combined treatment with tyrosine kinase inhibitors (TKi) and additional drugs is emerging as a promising strategy for cancer therapy. TKi and histone-deacetylase inhibitors (HDI) are two classes of anti-tumor agents with distant mechanisms of action. We have designed and synthesized chimeric compounds, which comprise structural elements of the TKi imatinib, and of prototypical HDI compounds. These compounds retain TKi activity similar to imatinib, exemplified by the inhibition of the platelet-derived growth factor receptor, and c-Kit kinase in intact cells. In addition, the chimeric compounds have *in vitro* and cellular HDI activity, and potently inhibit growth of cancer cell lines, including that of imatinib-resistant cell lines. Chimeric molecules with combined TKi and HDI activity may simplify combination treatment and be applicable to overcome clinical resistance to TKi single-agent therapy. *Anti-Cancer Drugs* 21:759–765

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Introduction

The treatment of several types of human cancers with inhibitors of oncogenic tyrosine kinases (TKi) has been established in the clinic as a therapeutic modality over the last two decades [1]. Imatinib/Gleevec and other inhibitors that block the activity of the BCR-Abl fusion proteins and of the members of the platelet-derived growth factor receptor (PDGFR)-kinase family PDGFRα, PDGFRβ, and cKit are currently applied for the treatment of chronic myeloid leukemia, several rare types of leukemia, dermatofibrosarcoma protuberans, and gastrointestinal stromal tumors [2–4]. The possible application of this group of inhibitors in other types of cancer, such as glioma/glioblastoma, is being tested in preclinical and clinical studies [5–7]. These malignancies overexpress or harbor activated versions of the target kinases and depend, to a variable degree, on their activity. Clinical experience with these compounds showed several problems, most importantly primary or secondary resistance to treatment [8]. In both the cases, combination therapy of imatinib with classical cytostatic/cytotoxic drugs or new targeted agents may present a possible alternative [9]. One class of targeted drugs, which has been combined earlier with tyrosine kinase inhibitors, comprises inhibitors of histone deacetylases (HDI) [10]. HDI block histone deacetylation, but also interfere with reversible acetylation of other cell proteins like Stat 1 and 3, Hsp90 or α-tubulin. Therefore, this class of enzymes is acting as acetyl lysine-specific deacetylases, leading to a complex cellular response affecting gene expression, apoptosis regulation [11], and signal transduction in general. Notably, several HDI are potent, anti-proliferative, cytotoxic agents and have entered clinical trials for cancer treatment. An example is Vorinostat (Zolinza, SAHA), which has recently been approved for the treatment of cutaneous T-cell lymphoma [10,12]. Combination treatment of cancer cells in tissue culture with TKi and HDI was shown to have additive or even synergistic effects, which could be partially attributed to the reversible inhibition of the target kinase by the TKi on the one hand, and down-regulation of the target kinase expression by HDI on the other [13–15]. It is, however, likely that HDI exerts antitumor activity also through other pathways like the extrinsic apoptosis pathway as described in acute myeloid leukemia [10], which may likewise lead to synergy with TKis [16]. We describe here the combination of both the principles – inhibition of tyrosine kinases of the PDGFR family and of histone deacetylases – in one and the same compound. The new chimeric molecules retain activity towards both the targets, potently inhibit proliferation of PDGFR-dependent cell lines, and also inhibit proliferation of imatinib-resistant glioma cell lines. Chimeric TKi/HDI molecules may be more generally applicable to overcome resistance to common tyrosine kinase inhibitors.

Materials and methods

Chemical synthesis of the chimeric TKi/HDI has been described elsewhere [17]. Imatinib/STI571 was synthesized as described earlier [18]. Trichostatin A (TSA,

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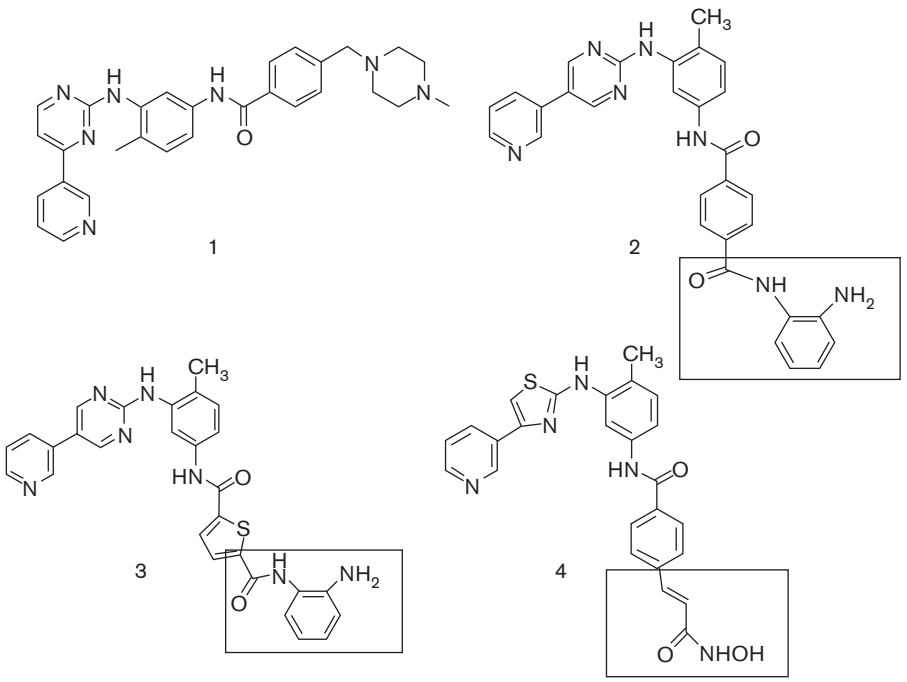
[*R*-(*E*,*E*)]-7-[4-(dimethylamino)phenyl]-*N*-hydroxy-4,6-dimethyl-7-oxo-2,4-heptadienamide) was obtained from Sigma-Aldrich (Taufkirchen, Germany). BA/F3 cells expressing a TEL-PDGFβ-receptor fusion protein were provided by Dr Donald Small (Johns Hopkins University, Baltimore). They were cultivated in RPMI1640 medium containing 10% heat-inactivated fetal bovine serum and 1 ng/ml murine recombinant interleukin 3 (IL-3) (Peprotech, London, UK) at 37°C, in a humidified atmosphere with 5% CO_2. The proliferation was determined using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. BA/F3 cells were washed twice with PBS, and 1×10^5 cells in 200 µl culture medium were seeded into 96-well microtiter plates and incubated in the absence or presence of the compounds (final concentration of dimethyl sulfoxide 0.1%) for 72 h. Thereafter, 20 µl of MTT solution (5 mg/ml in PBS) was added, and the cells were incubated for another 4 h. Fifty microliters of solubilization reagent (20% SDS, 0.02 mol/l HCl) were then added, the plates incubated at 37°C overnight, and absorption at 570 nm was subsequently measured using a microplate reader. The diagrams represent mean values (± standard error of mean) of eight parallel samples. All experiments were repeated at least three times and the representative data sets are shown. The glioma cell lines were provided by Professor Monica Nistér, Cancer Center Karolinska, Stockholm. They were seeded at a density of 4×10^3 cells per well of the 96-well plates. The next day, the compounds were added (final concentration of dimethyl sulfoxide 0.1%) and incubation was carried out for 3 days before proliferation was assessed with MTT as described above. Swiss 3T3 cells were seeded at a density of 2×10^3 cells per well in 96-well plates in Dulbecco's modified Eagle's minimal essential medium, containing 10% fetal bovine serum. The next day, the medium was changed to Dulbecco's modified Eagle's minimal essential medium with 2% FCS, with or without 20 ng/ml of human recombinant platelet-derived growth factor (PDGF-BB), and the test compounds were added. Cell proliferation was assayed with MTT as described above after 2 days. For assessing histone acetylation, tubulin acetylation, and expression of p21 or p27, the cells were treated for 24 h and subsequently lysed in lysis buffer (50 mmol/l Tris–HCl, pH7.5, 150 mmol/l NaCl, 1% NP40, 0.5% deoxycholate, 0.2% SDS, 1 mmol/l sodium vanadate, 20 mmol/l NaF, 1 mmol/l phenylmethylsulfonylfluoride, 1 µg/ml leupeptine, 1 µg/ml pepstatin), supplemented with 2 µl benzonase (VWR International, Darmstadt, Germany) per ml. Equal amounts of lysate protein (determined with the BCA assay, Thermo Fisher Scientific Bonn, Germany) were separated with 15% SDS-PAGE gels, transferred to PVDF membranes, and histone acetylation was detected using anti-AcH3 (Lys9 + 14) rabbit polyclonal antibody (Calbiochem, Bad Soden, Germany, cat. # 382158), anti-AcH4 antibodies (TP25, kindly provided by Dr Oliver Krämer, Institute of Biochemistry, University of Jena, Germany), anti-acetyl tubulin (sc-23950), anti-p21(sc-6246), or anti-p27 (sc-1641) antibodies (all from Santa Cruz, Heidelberg, Germany). Phosphorylation of PDGF receptors in Swiss 3T3 cells, of cKit in CHRF cells, and of Bcr-Abl in K562 cells was analyzed as described earlier [19,20].

Results and discussion

The compounds designed were harboring the *N*-(3-(4-(pyridin-3-yl) pyrimidin-2-ylamino) phenyl)amide pharmacophore core of imatinib [2] (Fig. 1), and either the benzamide (compound 2, 3) or the hydroxamic acid (compound 4) head group (indicated in Fig. 1) as Zn^{2+} complexing motifs of the commonly used HDAC inhibitors such as MS-275, or suberoylanilid hydroxamic acid (SAHA) and TSA [21], respectively. Additional details of the structural considerations and chemical synthesis of these compounds are described elsewhere [17].

In the initial studies, cytotoxicity towards the EOL1 cell line isolated from a patient with hypereosinophilic syndrome bearing a constitutively active FIP1L1-PDGFRα fusion protein [22] was evaluated. These cells are known to depend for proliferation on the activity of this imatinib-sensitive fusion protein. Notably, compounds 2–4 displayed a very high cytotoxicity towards EOL1, comparable with that of imatinib (Table 1), and consistent with their capacity to inhibit recombinant PDGFR *in vitro* (Table 1). In contrast, SAHA, which has no TKi activity, is comparatively ineffective in inhibiting EOL1 cell proliferation (Table 1). Next, we subjected the chimeric compounds to growth assays in BA/F3 cells. BA/F3 is a murine pro-B cell line, which depends for survival and proliferation on the cytokine IL-3) [23]. IL-3 stimulates proliferation of these cells through a mechanism involving the activity of the Janus kinase 2 [24]. The cells can be made IL-3 independent by stable transfection with activtated tyrosine kinases, such as the transforming version of the PDGFβ-receptor, TEL-PDGFRβ [25]. Selective inhibition of the PDGFR activity in these cells then leads to growth arrest and apoptosis. This can be rescued by the addition of IL-3, as Janus kinase 2 is resistant to most PDGFR inhibitors. The described inhibition pattern could be observed upon treatment of the cells with imatinib (Fig. 2a). The chimeric compounds 2–4 inhibited the growth of TEL-PDGFRβ expressing cells as well, but with somewhat lesser efficiency than imatinib. IL-3 rescued the inhibition partially, but less efficiently than for imatinib (Fig. 2b–d). Proliferation of the parental BA/F3 cells, which do not contain activated PDGF receptors, is entirely dependent on IL-3 (Fig. 2e). Again, growth in the presence of IL-3 can be inhibited with the chimeric compounds (example shown for compound 3, Fig. 2e). These effects on BA/F3 cells suggested that growth inhibition by the chimeric molecules was partially mediated by a mechanism, which is distinct from PDGFR inhibition. As the compounds are reasonable (compounds 2, 3), or even relatively potent (compound 4) HDI *in vitro* (Table 1), it was considered

Fig. 1



Structures of compounds used in this study. (1) imatinib (STI571), (2) (example 12b in [17]), (3) (example 14b in [17]), (4) (example 18b in [17]) chimeric compounds containing the pharmacophore of imatinib (2, 4) or a modification of it (3) and either a benzamide headgroup (2, 3) or a hydroxamate headgroup (4) for histone deacetylase inhibition. The HDI headgroups are framed.

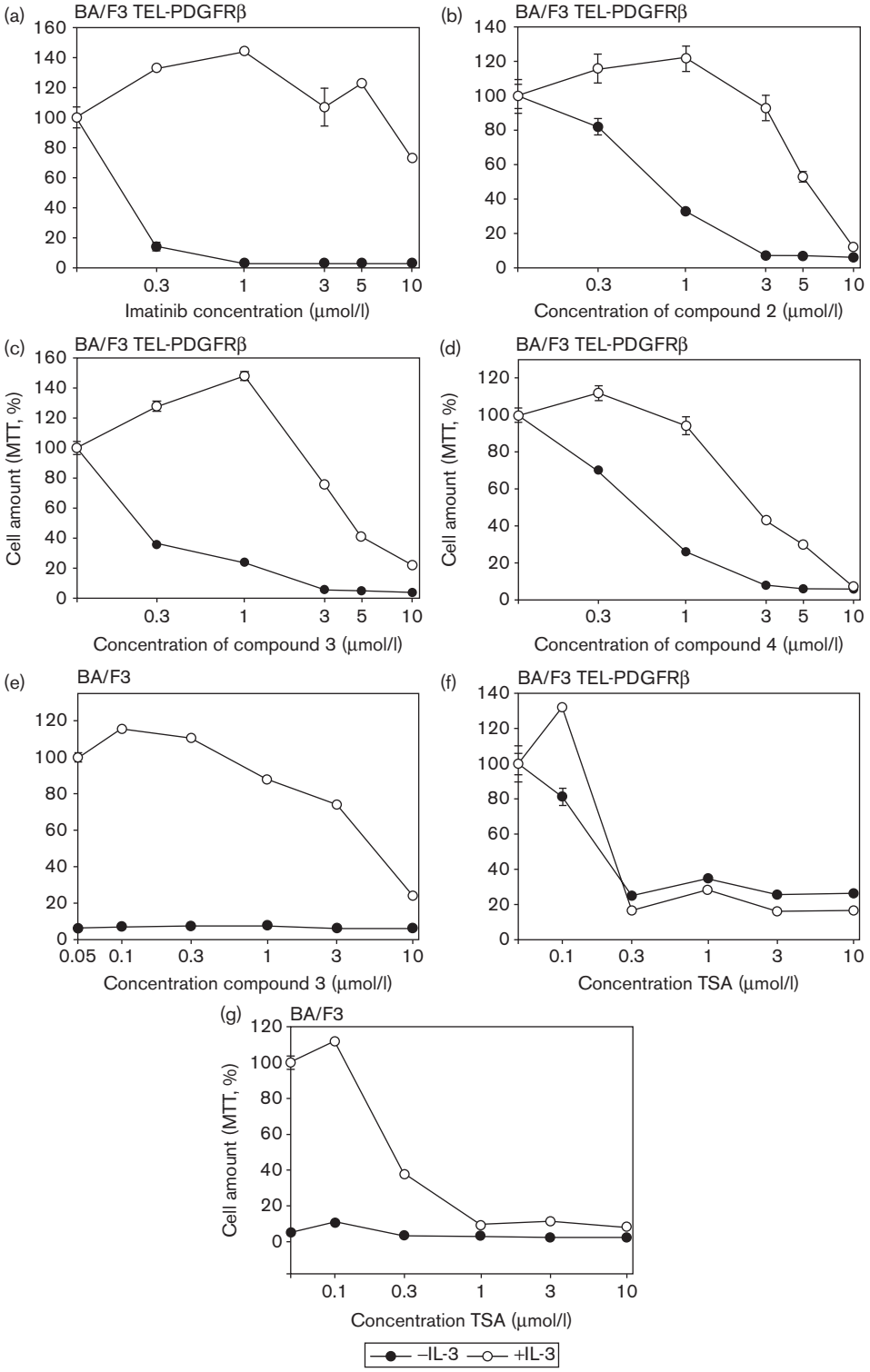
Table 1 Selectivity of chimeric tyrosine kinase – HDAC – inhibitors

Compound	Cytotox EOL1 IC_{50} (μmol/l)	rHDAC1 inhibition IC_{50} (μmol/l)	rHDAC6 inhibition IC_{50} (μmol/l)	rhPDGFRβ inhibition IC_{50} (μmol/l)	Cytotox A549 IC_{50} (μmol/l)	Cytotox HeLa IC_{50} (μmol/l)
Imatinib	0.0016	>100	>100	0.24	11.5	20
SAHA	1.0	0.02	0.052	ND	2.2	2.2
2	0.0072	0.27	>100	2.6	3.95	1.97
3	0.0015	0.23	>100	5.2	2.44	1.15
4	0.0058	0.08	0.41	3.9	6.85	4.65

Activity of the chimeric compounds 2–4 was compared with that of imatinib or the histone deacetylase inhibitor SAHA. The cytoxicity towards EOL1 HES, HeLa cervical carcinoma,and A549 NSCLC cell lines was assessed using the Alamar blue assay; recombinant HDAC enzymes and PDGFR were assayed *in vitro* (for experimental details see [17]). Compound designation in [17] is imatinib, 1; SAHA, 6; 2, 12b; 3, 14b; 4, 18b, respectively.
ND, not determined; PDGFR, platelet-derived growth factor receptor; SAHA, suberoylanilide hydroxamic acid.

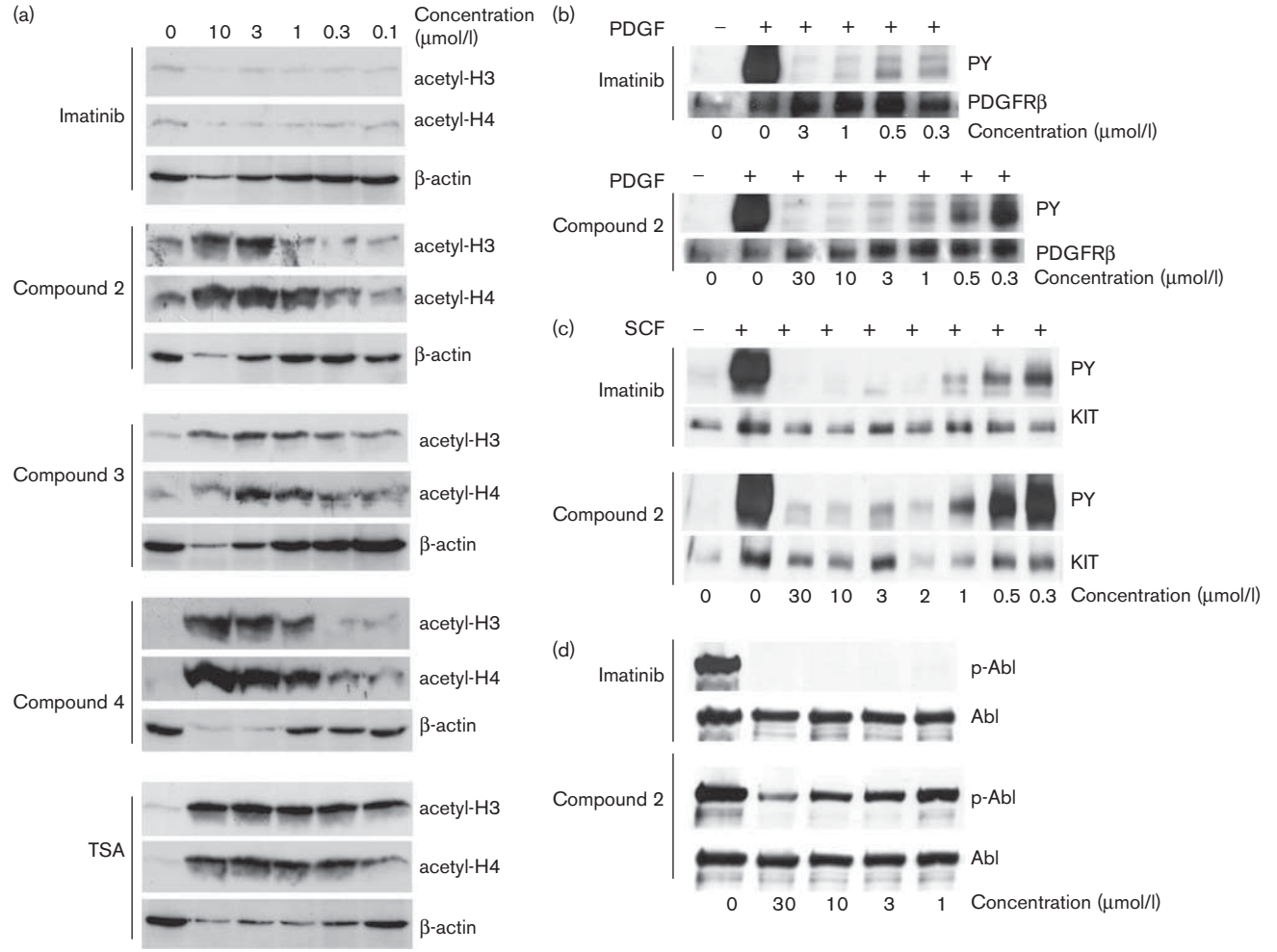
that this activity would form the basis for the inhibition of BA/F3 proliferation in the presence of IL-3. Indeed, treatment with the chimeric compounds but not with imatinib stimulated histone hyperacetylation in BA/F3 cells, as detectable by immunblotting with acetyl-histone H3-selective, and H4-selective antibodies (Fig. 3a). Treatment of the cells with TSA, a potent inhibitor from the hydroxamate family, was carried out for comparison. The potency of compounds 2 and 3 in this assay was clearly lower than that of TSA. Compound 4 was more potent than the other chimeric compounds in this cellular assay and nearly matched the activity of TSA, notably in the stimulation of histone H4 acetylation, consistent with its greater potency in vitro (Table 1). Growth of TEL-PDGFRβ expressing cells and of parental BA/F3 cells was potently inhibited by TSA, with little difference in the absence or presence of IL-3 (Fig. 2f and g), consistent with potent HDAC inhibition (and not PDGFR inhibition) forming the basis for anti-proliferative activity in this case. TSA also potently stimulated tubulin acetylation in BA/F3 cells, as has been observed earlier in other cells [26], whereas the chimeric compounds were inactive in this assay (not shown). Acetylated tubulin is a substrate of HDAC6 [26]. Tubulin deactylation is mediated by a site of HDAC6 designated the 'tubulin deacetylase (TDAC)' site, which is structurally and functionally distinct from the histone dacetylating site [27]. Compounds 2 and 3 were inactive against HDAC6 *in vitro*, but compound 4 inhibited HDAC6 *in vitro* in an assay assessing HDAC (but not TDAC) activity (Table 1). Cell treatment with this compound did, however, not cause tubulin acetylation. It can be inferred that although the

Fig. 2



Inhibition of cell proliferation of BA/F3 cells. The cells were treated with the different compounds at the concentrations indicated, in the absence or presence of interleukin-3 (IL-3), and proliferation/survival was determined using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. (a–d) Chimeric compounds were tested on TEL-platelet-derived growth factor receptor (PDGFRβ) expressing BA/F3 cells. For comparison, trichostatin A (TSA) was tested under the same conditions (f). Cell amounts were normalized to values for untreated cells under each of the conditions. (e and g) Effects of compound 3 or TSA on parental BA/F3 cells in the absence or presence of IL-3 is shown for comparison. Cell amounts were normalized to values for untreated cells in the presence of IL-3.

Fig. 3



Effect of chimeric compounds on signal transduction. (a) Histone acetylation was assessed in TEL-platelet-derived growth factor receptor (PDGFβ)-receptor expressing BA/F3 cells by immunoblotting of cell lysates and detection with anti-acetyl-histone H3 or anti-acetyl-histone H4, as indicated. Equal lysate protein amounts were loaded. Loading was additionally assessed by probing the blots for β-actin. Note that the highest compound concentrations led to cell loss that was not entirely compensated by loading as judged by actin signals. (b) Effect of imatinib and compound 2 on PDGF-receptor phosphorylation in Swiss 3T3 cells. Lysates of PDGF-stimulated cells (+) or unstimulated cells (−) were subjected to immunoblotting and detection of phosphotyrosine (PY) or PDGFβ-receptor, as indicated. (c) Effect of imatinib and compound 2 on cKit/ stem-cell factor (SCF)-rceptor phosphorylation in CHRF cells. Lysates of SCF-stimulated cells (+) or unstimulated cells (−) were subjected to the enrichment of glycoproteins with wheat-germ agglutinin beads. Extracted glycoproteins were then analyzed by immunoblotting and detection of PY or cKit/SCF-receptor, as indicated. (d) Effect of imatinib and compound 2 on Bcr-Abl phosphorylation in K562 cells. Cell lysates were subjected to immunoblotting with antibodies against phosphorylated Abl kinase (p-Abl), or Abl protein for loading control. SCF, stem cell factor; TSA, trichostatin A.

Zn-binding hydroxamic acid motif in this compound would allow TDAC inhibition, the other structural part of compound 4, the TKi moiety seems to confer selectivity for the HDAC site of HDAC6. Similar findings have been reported earlier for other HDAC inhibitors of the hydroxamic acid class [27]. In BA/F3 cells, neither of the compounds including TSA caused induction of p21, a frequently induced gene upon HDAC inhibition [28,29]. The specific treatment conditions may not have allowed detection of p21 induction, or induction of this gene may not be critical for the observed growth inhibition of this particular cell line. We detected, however, a weak elevation of p27, which has been described to be induced posttranscriptionally by HDIs [29,30], by compounds 2 and 3 (data not shown). Further research is required to identify target genes for the HDI activity of the chimeric compounds, which may mediate proliferation arrest.

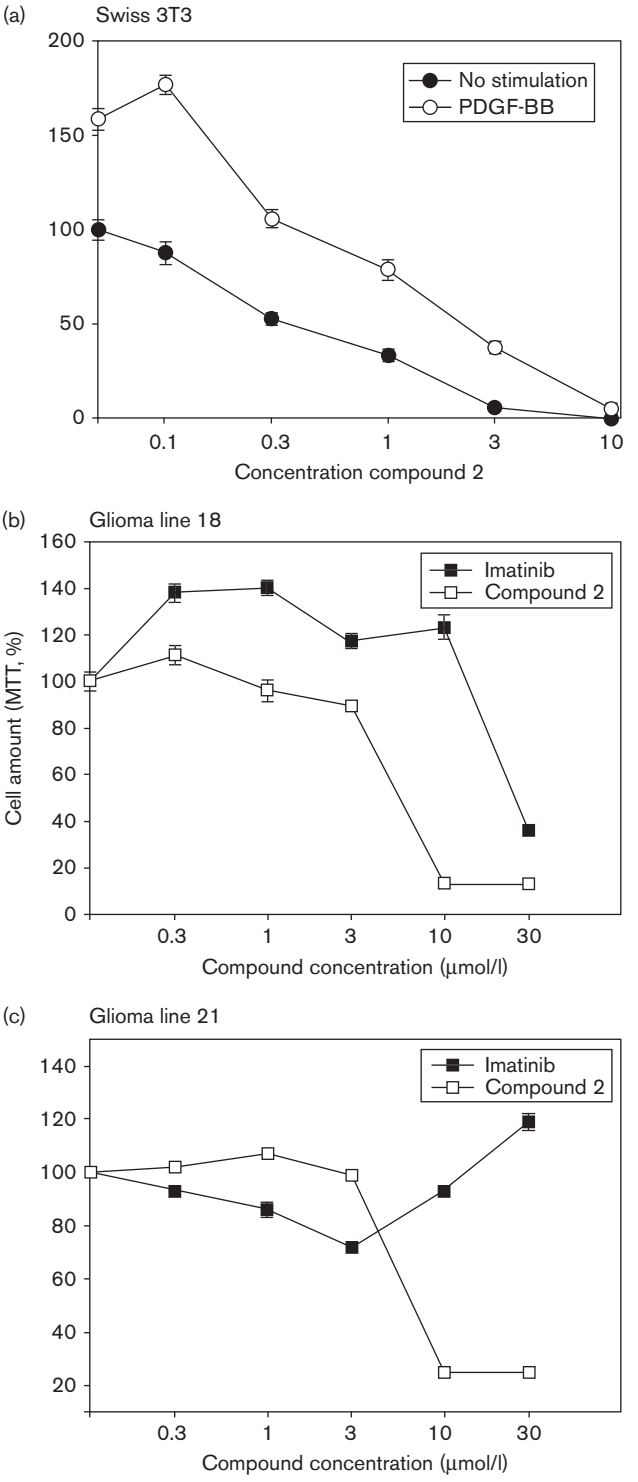
To characterize the cellular TKi activity of the compounds in more detail, we tested the inhibition of known target kinases of imatinib by compound 2, which was most potent against PDGFR kinase *in vitro* (Table 1). For this purpose, we first chose the mouse fibroblast line Swiss 3T3 and the human leukemia cell line CHRF, which express endogenously high levels of PDGF receptors and c-Kit, respectively. To activate these

kinases, the cell lines need to be stimulated with PDGF or stem-cell factor (SCF, the ligand of c-Kit) respectively. As shown in Fig. 3b and c, compound *2* inhibited cellular PDGFR and c-Kit only slightly less potently than imatinib. Another important target kinase for imatinib is the BCR-Abl fusion protein, which drives proliferation of chronic myeloid leukemia cells [31]. K562 cells express constitutively active and autophosphorylated BCR-Abl at relatively high levels, and were therefore chosen to test the cellular activity of compound *2* against this kinase. As shown in Fig. 3d, the chimeric compound was nearly inactive toward cellular BCR-Abl. Thus, in the chimeric compounds, the inhibitory activity towards tyrosine kinases is preserved; however, the selectivity profile seems somewhat modified. In biochemical assays, compound *2* was inactive towards protein kinase A up to 100 µmol/l, but Abl (wt), Abl mutant $T^{315}I$, PDGFRß, and VEGFR2 were inhibited between $IC_{50} = 2.6$ to 9.8 µmol/l (for a more detailed discussion including molecular modeling see [17]). Importantly, however, the compounds do not acquire activity against distantly related protein kinases such as protein kinase A.

We also tested anti-proliferative activity of compound *2* against the Swiss 3T3 cell line in the presence and absence of PDGF. The compound inhibited both PDGF-dependent and PDGF-independent growth of cells, consistent with its dual activity for inhibition of PDGFR kinase and histone deacetylases (Fig. 4a). Human gliomas often express PDGFα-receptors [32,33]. However, based on the studies in glioma-derived cell lines, only a subset of gliomas is sensitive to imatinib [5]. We, therefore, explored whether glioma cell lines, which were identified earlier as imatinib-resistant, would be inhibited by the chimeric compounds. As shown in Fig. 4b and c, this was indeed the case. Both the tested compounds *2* and *3* inhibited the growth of gliomas 18 and 21 [5], which were refractory to imatinib. Similarly, the compounds also inhibited the growth of two other cancer cell lines, HeLa cervical carcinoma and A549 non-small cell lung cancer, which do not harbor imatinib-sensitive tyrosine kinases, and are therefore also refractory to imatinib. Compounds *2*–*4* were, however, similarly active in the inhibition of these cells as the reference compound SAHA (Table 1).

In conclusion, chimeric compounds composed of core structures of imatinib and head groups of the commonly described HDIs retain TKi and HDI activity and anti-proliferative activity. Imatinib-resistant glioma cell lines are sensitive to chimeric compounds. The compounds described here are somewhat less potent for the inhibition of either tyrosine kinases or histone deacetylases than the reference compounds that are specific inhibitors of either enzyme class, and the selectivity profile in the cellular activity of the chimeric molecules is modified as compared with the parent compounds. Notably, the TKi moiety seems to confer selectivity for the HDAC activity as

Fig. 4



Chimeric tyrosine kinase-HDAC-inhibitors block proliferation of Swiss 3T3 cells and of imatinib-resistant glioma cells. (a) Swiss 3T3 fibroblasts were treated in the absence or presence of 20 ng/ml platelet-derived growth factor (PDGF)-BB with compound 2 for 2 days. (b and c) Imatinib-resistant glioma cell lines 18 (b) or 21 (c) were treated with the chimeric compound 2 or imatinib for 3 days. Proliferation was assessed with 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT).

opposed to TDAC activity of HDAC6 in the case of compound 4. Nevertheless, our data suggest that dual targeting of tyrosine kinases and of HDACs with a single compound is feasible. Compounds developed according to this strategy may simplify combined treatment exploiting both the principles of inhibition. They may particularly be suitable to treat TKi-resistant cancers.

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