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Short communication

4,5,6,7-Tetrahydro-isoxazolo-[4,5-c]-pyridines as a new class of cytotoxic Hsp90 inhibitors

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ABSTRACT

Hsp90 is considered an interesting therapeutic target for anticancer drug development. Here we describe a new class of 4,5,6,7-tetrahydro-isoxazolo-[4,5-c]-pyridine compounds. A small library of derivatives has been synthesized and investigated. Some reported compounds show interesting properties combining both notable binding to Hsp90 and potent cell growth inhibitory activity. N-5 substitution with a 2,4 resorcinol carboxamide appears crucial for activity. Moreover, a derivative bearing a hydroxamic acid residue bound to C-3 amide portion was found to inhibit both Hsp90 and HDAC6.

1. Introduction

Heat shock protein 90 (Hsp90) is a molecular chaperone protein that plays a key role in the conformational maturation, stability and function of so-called “client” proteins [1,2]. Many Hsp90 client proteins are over-expressed in cancer, often in mutated forms, and are responsible for unrestricted cell proliferation and survival. Inhibition of the ATPase activity of Hsp90 disrupts an ongoing “folding” cycle, involving multiple co-chaperone proteins and, in turn, leads to destabilization, ubiquitination, and ultimately proteasome-mediated degradation of client proteins, causing loss of function and inhibition of cell growth [3,4].

Hsp90 is normally expressed in healthy cells representing the 1–2% of the total intracellular proteins, from which about 3% is found in the nucleus with effects on the regulation of several nuclear events. In case of tumor tissues, however, its levels increase up to 4–6% of the whole proteomic load of the cell [5,6]. Interestingly, Hsp90 derived from tumor cells has particularly strong ATPase activity with higher binding affinity to inhibitors than the latent form in normal cells, allowing a possible selective targeting to tumor tissues [7].

Hsp90 has thus emerged as an important target in several diseases [8–11]. In particular, Hsp90 is considered an intriguing therapeutic target for anticancer drug development because its single inhibition represents an attack on multiple hallmark traits of cancer.

A number of highly specific Hsp90 inhibitors have been identified [12]. They redirect Hsp90 chaperoning activity and decrease cellular levels of its numerous cancer-related client proteins [6]. Such inhibitors exhibit promising antitumor activity as single agents or in combination with other cytotoxic agents, and some of them are in clinical development [8,13,14].

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The first-in-class Hsp90 inhibitor to enter clinical trials was the 17-allylamino analogue of geldanamycin (17-AAG, **1**, Fig. 1) [15]. However, despite a high in vitro activity, its interest was shadowed by poor water solubility, coupled to hepatotoxic side effects. Some of these problems have been partially solved by the discovery of 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG, **2**) derivative. Radicicol (**3**), a natural macrocyclic antifungal antibiotic, was found to inhibit Hsp90 protein by interacting with the same site of action of geldanamycin [16]. However, due to its intrinsic chemical instability, it is devoid of in vivo activity.

Some synthetic compounds containing the isoxazole nucleus (**4**–**6**) have shown potent and selective inhibition of Hsp90 [17–20]. The presence of the heterocyclic nucleus seems to exert a key role in the docking of these compounds to the ATP-binding site of Hsp90 [21].

In this context, the 4,5-diarylisoxazole scaffold demonstrated of remarkable importance, recently leading to **5**, currently in Phase II clinical trials [17]. Additionally, the benzoisoxazole derivative **4**, having a resorcinol moiety in position 3, has also been disclosed as potent Hsp90 inhibitor [18].

However, to date, no Hsp90 inhibitors in clinical trials fully satisfy requisites of safety and stability. Therefore, finding further potent and selective Hsp90 inhibitors still remains an interesting and promising goal [22].

Recently, a structural investigation on the isoxazole scaffold led us to the discovery of 3,4-isoxazolediamide compounds, with a nitrogen atom directly attached to the C-4 heterocycle ring, endowed with potent Hsp90 inhibitory properties. Several derivatives from this series combine potent binding and cell growth inhibitory activities. In vivo studies proved also important antitumor effect of the morpholine-bearing derivative **6** against human epidermoid carcinoma A431 [20].

We envisaged that isoxazole structure could constitute a portion able to contribute to a high activity in various scaffolds, also taking into consideration other derivatives from previous literature. Structures described in recent papers [23–25], containing condensed bicyclic groups (as isoindoline or tetrahydroisoquinoline), have been thus considered in order to build a novel similar series of potential Hsp90 inhibitors.

Table 1
Binding on Hsp90 (FP Assay) and cytotoxicity on NCI-H460 non-small cell lung carcinoma cells.

Compound	NCI-H460 (IC_{50}; μM)	Hsp90 (FP) (IC_{50}; μM)
5	0.0024	0.061
11	>1	>100
13	>20	0.16
14	>20	3.9
15	0.87	0.1
17	9.7	0.11
18	>20	1.2
19	0.45	0.029
20	>1	>100
21	>1	>100
22	>20	1.4
23	0.23	–
25	0.19	0.054
26	0.9	0.068
27	0.2	0.034
29	3.9	0.033

So, herein we will describe the synthesis and Hsp90 inhibitor activity of a new class of 4,5,6,7-tetrahydro-isoxazolo-[4,5-c]-pyridine compounds **7**. A small library of different N-5 amide derivatives were synthesized and investigated. The resorcinol portion was firstly considered of importance by virtue of its role in the interaction with the Hsp90 protein either in radicicol and other synthetic series of compounds [20,23–26]. However, structural alterations and substitution of the resorcinol group was also investigated, as well as different N-substitutions at the C-3 amide portion.

Some of the synthesized compounds showed interesting activity combining both notable binding properties and potent cell growth inhibitory activity.

In the meantime, considering the efforts and the interest recently devoted by some of us into HDAC inhibitors field [27–29], we envisaged a strategy taking into consideration the key role of

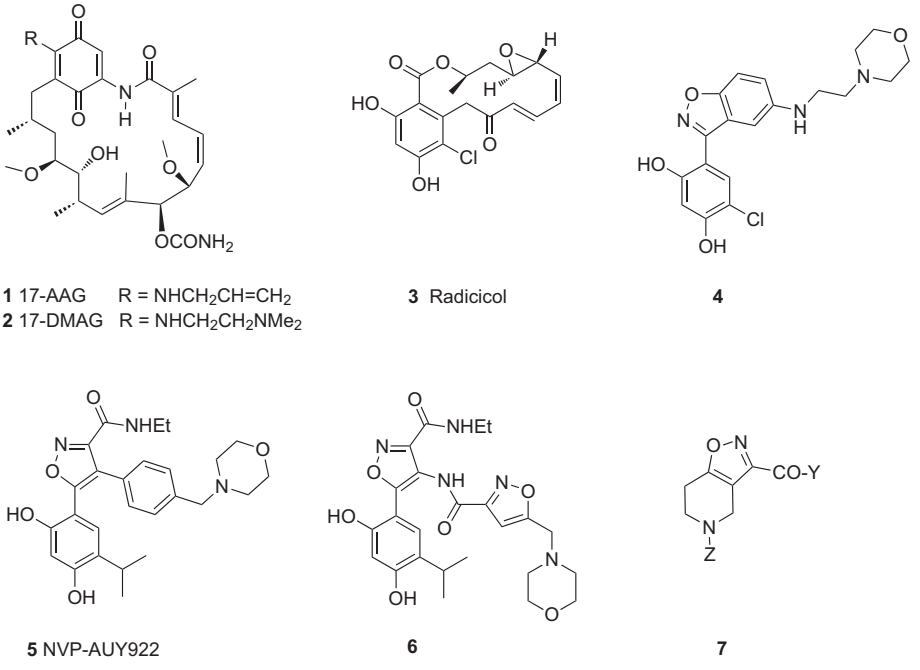


Fig. 1. Known Hsp90 inhibitors (**1**–**6**) and the new scaffold **7**. Distinct Y and Z refer to compounds detailed in Scheme 1.

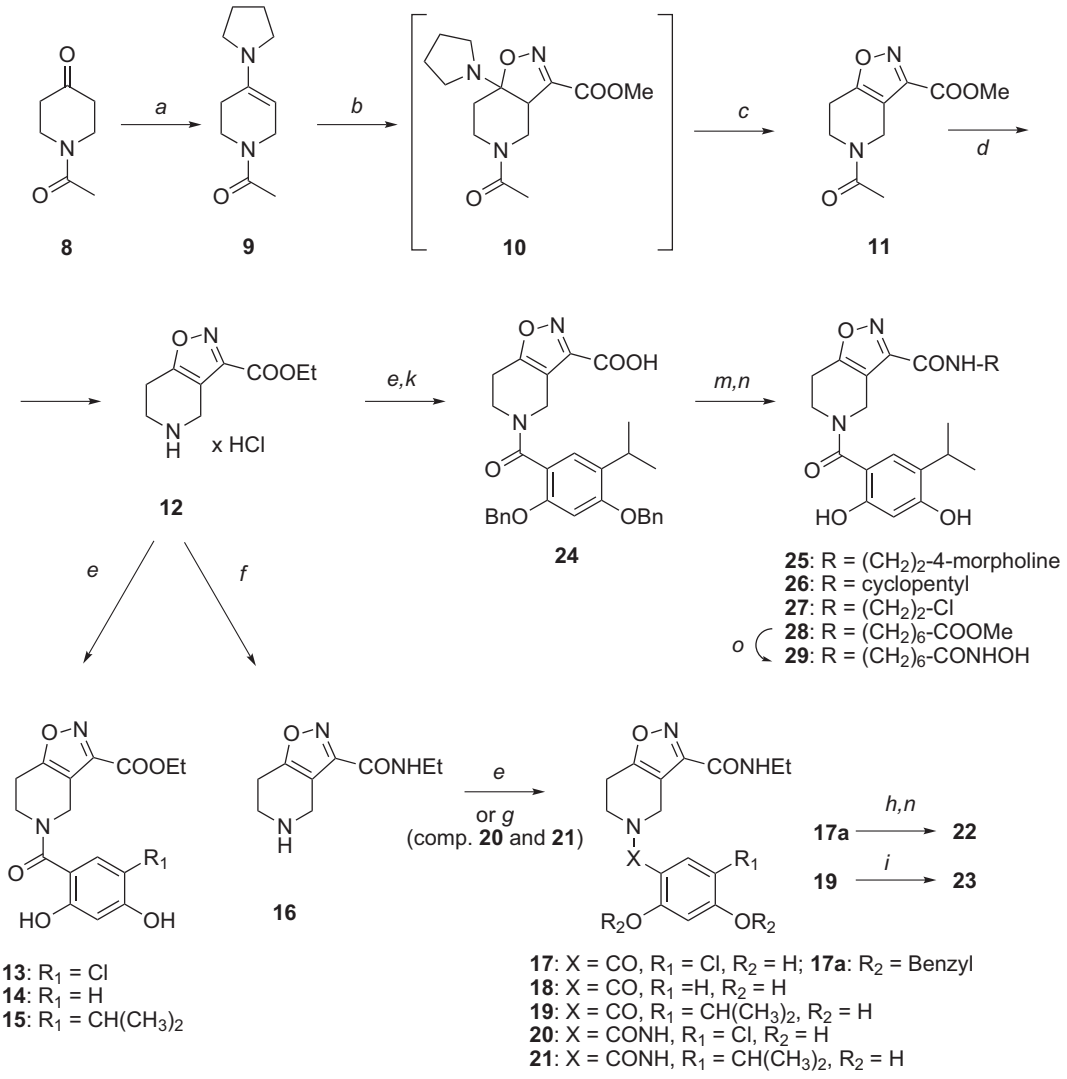
Hsp90 deacetylation performed by HDAC6 as a crucial step to ensure its chaperon activity [30–32]. In the present series, various C-3 amide N-substitutions demonstrated to maintain appreciable binding and growth inhibition activity, so we tried to add in this position a long chain hydroxamic acid residue (derivative **29**) in order to possibly obtain a Hsp90/HDAC dual inhibitor.

2. Chemistry

The synthetic route used for the preparation of the esters **13**–**15** and amides **17**–**21** (Table 1) is shown in Scheme 1. The 1,3-dipolar cycloaddition of nitriloxide, generated in situ by treating the methyl chlorooximineacetate with triethylamine in dichloromethane (DCM), to the pyrrolidine-enamine derivative **9**, is a known reaction that has been exploited for the synthesis of isoxazole-fused piperidine derivatives [33]. The enamine **9** was obtained from the N-acetyl-piperidone **8** and pyrrolidine in toluene at reflux.

The isoxazoline intermediate **10** was treated with trifluoroacetic acid (TFA) in DCM at reflux for 8 h affording the 4,5,6,7-tetrahydro-isoxazole-[4,5-c]-pyridine derivative **11**, in turn hydrolyzed with 32% hydrochloric acid to give the amine ethyl ester **12**. Reaction of the latter with the opportune carboxylic acid in the presence of 1-hydroxybenzotriazole (HOBt) and N′-(3-dimethylaminopropyl)-N-ethyl-carbodiimide hydrochloride (EDC) in DMF gave easily the desired **13**–**15**. Alternatively, the amine **12** was reacted with ethylamine in methanol at reflux to give in good yield the ethyl amide **16**. The desired amides **17**–**19** were obtained by condensation of **16** with the proper carboxylic acid in the same manner as described previously for compounds **13**–**15**. Otherwise, compound **16** was reacted with opportune (commercial or synthesized from arylamine precursor) isocyanate to give ureas **20** and **21**. The methylene derivative **22** was obtained by the selective reduction of **17a** (the protected 2,4 dibenzylated analog of **17**) using $LiAlH_4$ in THF; whereas **23** was simply obtained by reacting **19** with acetic anhydride in DCM solution.

Finally, derivatives **25**–**28** were prepared by reaction of the carboxylic acid derivative **24,** in turn obtained from the piperidine **12** by condensation with the bis-2,4-benzyloxyi-5-isopropylbenzoic acid, with appropriate amine using benzotriazolyl-1-oxy-tripyrrolidino-phosphonium hexafluorophosphate (PyBOP) as condensing agent and diisopropylethylamine (DIPEA). Hydroxamic derivative **29** was quantitatively prepared starting



Scheme 1. *Reagents and conditions*. **a**) pyrrolidine, p-TosOH, rfx; **b**) 2-chlorohydroxyimino acetic acid methyl ester, TEA, DCM; **c**) TFA, DCM, rfx; **d**) HCl 32%, EtOH, rfx; **e**) HOBt, WSC, opportune acid, DMF; **f**) EtNH2 in MeOH, rfx; **g**) Step 1: 5-chloro-2,4-dimethoxyphenyl isocyanate, 1,4 dioxan; step 2: BBr_3 1 M in DCM, for comp. **20**; step 1: 2,4-bis-(benzyloxy)-5-isopropylaniline, trichloromethyl chloroformate, 1,4 dioxan, then **16**; step 2: BCl3 1 M in DCM for comp. **21**; **h**) LiAlH4, THF; **i**) Ac_2O, DMF; **k**) LiOH, THF/H_2O; **m**) PyBOP, DIPEA, opportune amine; **n**) BCl_3 1 M in DCM; **o**) NH_2OH, MeOH, NaOH*aq*.

from the corresponding methyl ester intermediate **28** by reaction with hydroxylamine in MeOH in the presence of aq. NaOH [28].

Removal of benzyl or methyl protecting groups at phenolic functions were routinely conducted with good yields using boron trichloride or boron tribromide, respectively, in DCM.

3. Biological results and discussion

Measurements of binding by a fluorescence polarization (FP) assay and inhibition of cell growth proliferation of NCI-H460 tumor cell line were determined for all the synthesized compounds. Flow cytometric analysis of cell cycle was also determined for two representative compounds (**17** and **19**). Among the group of esters **13**–**15**, the isopropyl resorcinol derivative **15** was the most potent compound in terms of antiproliferative activity and affinity to HSP90α (Table 1); whereas, compounds **13** and **14**, despite retaining some binding ability, resulted devoid of cellular activity. Among the group of the ethylamides **17**–**23**, compound **19**, along with an important binding property, showed a notable antiproliferative activity. Otherwise, the chlororesorcinol derivative **17** and compound **18** resulted devoid of cytotoxicity. Quite surprisingly, compounds **20** and **21**, where the N-5 amide portion was elaborated into an ureidic moiety resulted with no activity. Detrimental for activity resulted also the reduction of the N-5 amide moiety in compound **22**.

Compound **25**–**27,29** modified at the C-3 amide portion were synthesized and screened to better substantiate our structure–activity investigation. Interestingly, the morpholine water solubilizing group in compound **25** was well tolerated retaining good binding and cell growth inhibiting properties. The cyclopentyl and chloroethyl derivatives **26** and **27** as well as the hydroxamic derivative **29** also retained binding and cell growth activity. Of interest, compounds **25** and **27** showed stronger antiproliferative activity than the starting ethyl amide derivative **19** indicating that the C-3 amide region could be of some importance in modulating the activity of this class of compounds. Of some interest resulted also the ester derivative **23**, a diacetyl prodrug of **19**, whose cellular activity result increased as compared to **19** itself notwithstanding the drop of binding capability. Additionally, cell viability, apoptosis and flow cytometric analysis of cell cycle was determined for compounds **17** and **19** in comparison with 17-AAG. **17** and **19** elicited a concentration-dependent growth inhibitory effect in K562 cells with an IC_{50} of 32.6 and 0.72 μM respectively after 72 h drug exposure (Table 2).

Furthermore, the flow cytometric analysis showed that both **17** and **19** determined an increase in the proportion of cells in the G1 phase of the cell cycle with a strong reduction of the S phase fraction (Fig. 2). This effect was similar to that observed in the cells exposed to reference compound 17-AAG (**1**) 1 μM and was in agreement with previous reports that demonstrate that although Hsp90 inhibitors can affect cell cycle progression at multiple levels, a G1 arrest is seen in the majority of cell lines, provided that an intact Rb pathway is present [34].

Table 2
Cytotoxic and apoptotic effects of the Hsp90 inhibitors **17** and **19** in K562 cells.

	IC_{50} (μM)	AC_{50} (μM)
17	32.6	>100
19	0.72	5.4
17-AAG	0.15	3.1

The cells were exposed to the drugs for 72 h. Cytotoxicity was evaluated by the Trypan blue dye exclusion test, while apoptosis was measured by fluorescence microscopy IC_{50}: growth inhibitory concentration inducing 50% apoptotic cell death. AC_{50}: concentration inducing 50% apoptotic cell death.

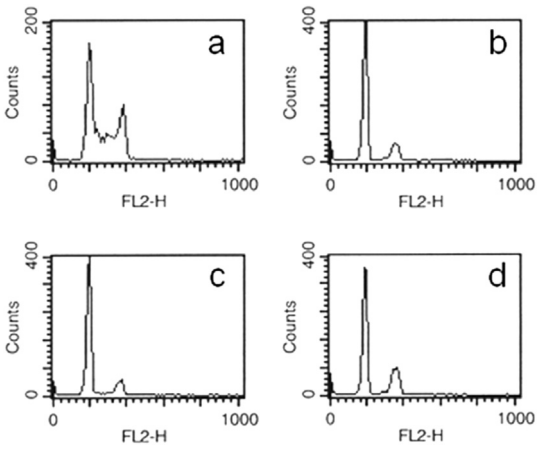


Fig. 2. Cell cycle distribution of K562 cells exposed to the Hsp90 inhibitors. The cells were exposed to the drugs for 24 h and cell cycle was evaluated by flow cytometry. (a) control untreated cells, (b) 17-AAG 1 μM, (c) **17** 75 μM, (d) **19** 10 μM. The data are representative of three separate experiments.

Finally, the compound **29** was investigated for the HDAC activity by measuring its potency in inhibiting 10 isolated human HDAC isozymes in the presence of a fluorogenic peptide bound to the RHKK(Ac), fragment of p53 (residues 379–392), and RHK(Ac)K(Ac) for HDAC8, as the substrate, and using SAHA as the reference compound (Table 3) [35].

In addition to being very active against Hsp90, in the HDAC profile **29** also showed a high selectivity towards the isoform HDAC6. This compound represents an interesting hit compound for further studies in the dual targeting HDAC/Hsp90 inhibition approach.

4. Conclusions

In summary, a novel class of 4,5,6,7-tetrahydro-isoxazolo-[4,5-c]-pyridine derivatives has been synthesized and tested for their affinity to Hsp90 and for their antiproliferative activity.

A group of compounds **13**, **15**, **17**, **19**, **22, 25**–**27, 29** showed binding affinity at micromolar/nanomolar level. Compounds **15, 19**, **23, 26**–**27, 29** showed also cytotoxic activity in sub-micromolar range (200–900 nM). The flow cytometric analysis showed that both **17** and **19** determined an increase in the proportion of cells in the G1 phase of the cell cycle with a strong reduction of the S phase fraction (Fig. 2). The effect was in agreement with that previously reported for Hsp90 inhibitors. Attempts to investigate structural alterations of the resorcinol portion, as well as of the N-5 amide moiety had only deleterious effect on activity. Conversely, structural alteration of the C-3 amide seems of importance in modulating the activity of the compounds.

Table 3
In vitro inhibitory activity ($IC_{50;}$ nM) of **29** vs. SAHA against isoforms HDAC1–8, 10–11.[a]

HDAC isoform	1	2	3	4	5	6	7	8*	10	11
29	2030	9490	4230	75900	1580	4.1	2510	1760	886	521
SAHA	258	921	350	493	378	28.9	344	243	456	362

[a] Compounds were tested in 10-dose IC_{50} mode, in triplicate with 3-fold serial dilution starting from 10 μM solutions. Isolated human HDAC isozymes were used in presence of a fluorogenic peptide bound to the RHKK(Ac) fragment, except for HDAC8*: where was used the diacetyl RHK(Ac)K(Ac), as substrate. Values are the means of three experiments and are given in nanomolar.

5. Experimental part

5.1. Chemistry

Reagents were purchased from commercial suppliers and used without further purification. 1H NMR spectra were recorded, unless otherwise indicated, in DMSO solution at 200 MHz on a Bruker AC-200, 300 MHz on a Varian Gemini-300, 400 MHz on a Varian Mercury Plus 400 and 500 MHz on a Varian Gemini-500 spectrometer, and peak positions are given in parts per million downfield from tetramethylsilane as the internal standard. *J* values are expressed in hertz. Electrospray mass spectra were recorded on a Waters Micromass ZQ-2000 instrument or a double-focusing Finnigan MAT 95 instrument with BE geometry. Thin layer chromatography (TLC) was carried out using Merck precoated silica gel F-254 plates. Flash chromatography was done using Merck silica gel 60 (0.063–0.200 mm). Solvents were dried according to standard procedures, and reactions requiring anhydrous conditions were performed under argon. Solutions containing the final products were dried with Na_2SO_4, filtered, and concentrated under reduced pressure using a rotatory evaporator.

5.1.1. N-Ethyl-4,5,6,7-tetrahydroisoxazole[4,5-c]pyridine-3-carboxamide (**16**)

Compound **12** (4.3 mmol, 1 g) was suspended in ethylamine 2 M in MeOH solution (51.7 mmol) and stirred for 12 h. The solution was concentrated in vacuo to give a residue which was purified by flash chromatography (DCM/MeOH 9.5/0.5). Yield: 65%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.23 (t, $J = 7.2$ Hz, 3H), 2.80–2.83 (m, 2H), 3.16–3.19 (m, 2H), 3.42–3.49 (m, 2H), 4.06 (s, 2H), 6.75 (br, 1H). MS (ESI): *m/z* 196.1 $[M+H]^+$.

5.1.2. General procedure for the N-acylation of 4,5,6,7-tetrahydroisoxazole[4,5-c]pyridines derivatives **12** and **16**

A mixture of the amine **12** or **16** (0.56 mmol), EDC × HCl (0.67 mmol, 128 mg), HOBt (0.67 mmol, 90,5 mg), TEA (1.7 mmol, 171 mg, 0.24 mL) and the opportune acid (0.56 mmol) in 10 mL of DMF was stirred at room temperature overnight and then the solvent was removed in vacuo. The residue was partitioned between AcOEt (15 mL) and 5% aq. citric acid (10 mL), the organic layer was separated and washed successively with saturated sodium bicarbonate solution (10 mL), brine (5 mL), dried over Na_2SO_4, filtered and evaporated in vacuo. The crude material was purified by flash chromatography (DCM/MeOH 9.5:0.5) to give the desired compound (**13**–**15**, **17**–**19**).

5.1.2.1. Ethyl 5-(5-chloro-2,4-dihydroxybenzoyl)-4,5,6,7-tetrahydroisoxazole[4,5-c]pyridine-3-carboxylate (**13**).

From **12** and 5-chloro-2,4-dihydroxybenzoic acid. (Yield: 72%) ^{1}H NMR (200 MHz, DMSO, 120 °C) δ 1.31 (t, $J = 7$ Hz, 3H), 2.89–2.95 (m, 2H), 3.77 (t, $J = 5.6$ Hz, 2H), 4.37 (q, $J = 7$ Hz, 2H), 4.57 (s, 2H), 6.57 (s, 1H), 7.11 (s, 1H). MS (ESI): *m/z* 366.9 $[M+H]^+$.

5.1.2.2. Ethyl 5-(2,4-dihydroxybenzoyl)-4,5,6,7-tetrahydroisoxazole[4,5-c]pyridine-3-carboxylate (**14**).

From **12** and 2,4-dihydroxybenzoic acid. (Yield: 40%) ^{1}H NMR (200 MHz, DMSO, 120 °C) δ: 1.30 (t, $J = 7$ Hz, 3H), 2.74 (s, 2H), 3.77 (t, $J = 5.6$ Hz, 2H), 4.35 (q, $J = 7$ Hz, 2H), 4.57 (s, 2H), 6.28–6.34 (m, 2H), 7.01 (d, $J = 8.2$ Hz, 1H), 7.95 (br, 1H), 9.22 (br, 1H), 9.37 (br, 1H); ^{13}C NMR (DMSO) δ: 13.8, 23.3, 61.8, 102.2, 106.8, 111.9, 114.2, 129.4, 129.8, 152.2, 155.2, 159.4, 159.6, 168.6, 169.3. MS (ESI): *m/z* 332.7 $[M+H]^+$.

5.1.2.3. Ethyl 5-(2,4-dihydroxy-5-isopropylbenzoyl)-4,5,6,7-tetrahydroisoxazole[4,5-c]pyridine-3-carboxylate (**15**).

From **12** and 5-isopropyl-2,4-dihydroxybenzoic acid. (Yield: 70%) ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.21 (d, $J = 6.8$ Hz, 6H), 1.38 (t, $J = 7.2$ Hz, 3H), 3.05 (t, $J = 5.6$ Hz, 2H), 3.16 (sept, 1H), 3.93–3.95 (m, 2H), 4.39–4.44 (m, 2H), 4.80 (s, 2H), 6.44 (s, 1H), 7.12 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 14.2, 22.8, 23.7, 26.4, 62.4, 104.2, 108.4, 110.3, 111.9, 126.7, 152.4, 157.9, 159.9, 168.8, 173.3. MS (ESI): *m/z* 375.3 $[M+H]^+$.

5.1.2.4. 5-(5-Chloro-2,4-dihydroxybenzoyl)-N-ethyl-4,5,6,7-tetrahydroisoxazole[4,5-c]pyridine-3-carboxamide (**17**).

From **16** and 5-chloro-2,4-dihydroxybenzoic acid. (Yield: 61%) ^{1}H NMR (200 MHz, DMSO, 120 °C) δ 1.14 (t, $J = 7.2$ Hz, 3H), 2.89 (t, $J = 5.8$ Hz, 2H), 3.26–3.36 (m, 2H), 3.76 (t, $J = 5.8$ Hz, 2H), 4.55 (s, 2H), 6.51 (s, 1H), 7.09 (s, 1H), 8.19 (br, 1H). MS (ESI): *m/z* 365.9 $[M+H]^+$.

5.1.2.5. 5-(2,4-Dihydroxy-benzoyl)-N-ethyl-4,5,6,7-tetrahydroisoxazole[4,5-c]pyridine-3-carboxamide (**18**).

From **16** and 2,4-dihydroxybenzoic acid. (Yield: 68%) ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.23 (t, $J = 7.2$ Hz, 3H), 3.01–3.04 (m, 2H), 3.43–3.47 (m, 2H), 3.93–3.96 (m, 2H), 4.92 (s, 2H), 6.35 (dd, $J = 8.4$ Hz, $J = 2.4$ Hz, 1H), 6.44 (d, $J = 2.4$ Hz, 1H), 7.26 (d, $J = 8.4$ Hz, 1H). ^{13}C NMR ($CDCl_3$) δ: 14.5, 23.3, 34.6, 40.8, 44.7, 104.2, 107.1, 108.5, 111.4, 130.2, 153.6, 159.4, 161.1, 162.1, 169.0, 173.1. MS (ESI): *m/z* 331.7 $[M+H]^+$.

5.1.2.6. 5-(2,4-Dihydroxy-5-isopropylbenzoyl)-N-ethyl-4,5,6,7-tetrahydroisoxazole[4,5-c]pyridine-3-carboxamide (**19**).

From **16** and 5-isopropyl-2,4-dihydroxybenzoic acid. (Yield: 68%) ^{1}H NMR (400 MHz, DMSO) δ 1.06–1.10 (m, 9H), 2.88 (t, $J = 5.6$ Hz, 2H), 3.03–3.09 (m, 1H), 3.23 (t, $J = 5.6$ Hz, 2H), 3.6 (br, 2H), 4.53 (s, 2H), 6.38 (s, 1H), 6.88 (s, 1H), 8.77 (br, 1H), 9.55 (s, 1H), 9.57 (s, 1H). ^{13}C NMR (DMSO) δ: 14.8, 23.0, 23.1, 24.5, 27.6, 35.2, 103.3, 112.5, 114.1, 127.4, 128.2, 154.9, 155.6, 159.0, 161.2, 169.9, 172.8. MS (ESI): *m/z* 374.1 $[M+H]^+$.

5.1.3. N^5-(5-Chloro-2,4-dihydroxyphenyl)-N^3-ethyl-6,7-dihydroisoxazolo[4,5-c]pyridine-3,5(4H)-dicarboxamide (**20**)

A solution of 5-chloro-2,4-dimethoxyphenyl isocyanate (0.28 mmol, 55 mg) and amine **16** (0.28 mmol, 60 mg) in dry dioxane (10 mL) was heated at 60 °C overnight. The reaction was cooled to room temperature, the solvent was removed in vacuo and the obtained residue was purified by flash chromatography on silica gel (hexane/AcOEt 3/7) to give N^5-(5-chloro-2,4-dimethoxyphenyl)-N^3-ethyl-6,7-dihydroisoxazolo[4,5-c]pyridine-3,5(4H)-dicarboxamide. Yield 73%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.25 (t, $J = 7.2$ Hz, 3H), 2.92–2.95 (m, 2H), 3.45–3.50 (m, 2H), 3.85–3.89 (m, 8H), 4.65 (s, 2H), 6.50 (s, 1H), 6.85 (br, 2H), 8.02 (s 1H). MS (ESI): *m/z* 408.9–410.1 $[M+H]^+$.

To a solution of this compound (0.12 mmol, 47 mg) in dry DCM (10 mL), cooled to −78 °C under argon, was added BBr_3 (1 M in DCM, 0.6 mmol, 0.6 mL) and stirring was continued for 30 min at the same temperature. The reaction was allowed to warm to rt and stirred for another 2 h, then saturated $NaHCO_3$ (5 mL) aqueous solution was added and DCM was removed in vacuo. The aqueous residue was extracted with AcOEt (3 × 10 mL), and the combined organic phases were washed with brine and dried. The crude residue obtained after evaporation was purified by flash chromatography on silica gel (DCM/MeOH 97/3). Yield 49%. ^{1}H NMR (DMSO, 400 MHz) δ 1.11 (t, $J = 7.2$ Hz, 3H), 2.88–2.91 (m, 2H), 3.25–3.28 (m, 2H), 3.75–3.77 (m, 2H), 4.53 (s, 2H), 6.45 (s, 1H), 7.21 (s, 1H), 8.05 (s 1H), 8.80 (br, 1H), 9.61 (s, 1H), 9.75 (s, 1H). ^{13}C NMR (DMSO) δ: 14.5, 23.1, 33.6, 104.1, 108.6, 111.3, 119.4, 125.7, 149.8, 150.1, 154.5, 155.9, 158.6, 168.6. MS (ESI): *m/z* 380.5–382.2 $[M+H]^+$.

5.1.4. N^5-(2,4-Dihydroxy-5-isopropylphenyl)-N^3-ethyl-6,7-dihydroisoxazolo[4,5-c]pyridine-3,5(4H)-dicarboxamide (**21**)

To a solution of 2,4-bis-(benzyloxy)-5-isopropylaniline [36] (0.58 mmol, 200 mg) in dry dioxane (15 mL) was added

trichloromethyl chloroformate (0.29 mmol, 57 mg, 0.036 mL), DIPEA (1.2 mmol, 154 mg, 0.208 mL) and the mixture was stirred at 60 °C for 4 h. The reaction was cooled to room temperature, **16** (0.58 mmol, 112 mg) was added and then heated at 60 °C overnight. The dioxane was evaporated and the crude residue was purified by flash chromatography on silica gel (hexane/ethyl acetate 9/1) to give N^5-(2,4-bis-(benzyloxy)-5-isopropylphenyl)-N^3-ethyl-6,7-dihydroisoxazolo[4,5-c]pyridine-3,5(4H)-dicarboxamide. Yield 52%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.21–1.28 (m, 9H), 2.86–2.89 (m, 2H), 3.34 (ept, $J = 6.8$ Hz, 1H), 3.44–3.51 (m, 2H), 3.81–3.84 (m, 2H), 4.63 (s, 2H), 4.99 (s, 2H), 5.06 (s, 2H), 6.56 (s, 1H), 6.79 (br, 1H), 6.92 (s 1H), 7.30–7.42 (m, 10H), 7.87 (s, 1H). MS (ESI): *m/z* 569.1 $[M+H]^+$.

To a solution of this compound (0.18 mmol, 100 mg) in dry DCM (10 mL) at −78 °C was added BCl_3 (1 M in DCM, 0.88 mmol, 0.88 mL) and stirring was continued for 30 min at the same temperature. The reaction was allowed to warm to rt and stirred another hour, saturated $NaHCO_3$ (5 mL) aqueous solution was added and DCM was removed in vacuo. The aqueous residue was extracted with AcOEt (3 × 10 mL), and the combined organic phases were washed with brine (5 mL) and dried. The crude residue obtained after evaporation was purified by flash chromatography on silica gel (hexane/AcOEt 1:1). Yield 64%. ^{1}H NMR (DMSO, 400 MHz) δ 1.08–1.11 (m, 9H), 2.90–2.92 (m, 2H), 3.06 (ept, $J = 6.8$ Hz, 1H), 3.24–3.29 (m, 2H), 3.76–3.80 (m, 2H), 4.55 (s, 2H), 6.31 (s, 1H), 6.88 (s, 1H), 8.16 (s 1H), 8.79 (br, 1H), 8.95 (s, 1H), 9.07 (s, 1H). MS (ESI): *m/z* 388.6 $[M+H]^+$.

5.1.5. 5-(5-Chloro-2,4-dihydroxybenzyl)-N-ethyl-4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridine-3-carboxamide (**22**)

To a suspension of $LiAlH_4$ (0.63 mmol, 100 mg) in dry THF (5 mL) was added a solution of 5-(5-chloro-2,4-dibenzyloxybenzoyl)-N-ethyl-4,5,6,7-tetrahydroisoxazole[4,5-c]pyridine-3-carboxamide **(17a)** (0.36 mmol, 200 mg) in dry THF (10 mL) over 10 min. The resulting mixture was stirred for 2 h, cooled in an ice-bath and the excess of hydride was decomposed by the dropwise addition of water followed by 15% sodium hydroxide and more water. After vigorous stirring for another 20 min the mixture was filtered with suction, the granular precipitate was washed with ethyl acetate. The filtrate was evaporated and the residue diluted water and extracted with AcOEt (2 × 15 mL). The organic phases were combined, dried, concentrated under vacuo and the residue was purified by flash chromatography (eluent: light petroleum/AcOEt 7/3). Yield 72%. MS (ESI): *m/z* 532,4–533,6 $[M+H]^+$.

70 mg of dibenzyl intermediate were deprotected with BCl_3 in the same way as described for compound **21**. Purification by flash chromatography (DCM/MeOH 95/5). Yield 45%, ^{1}H NMR (400 MHz, CD_3OD): δ 1.19 (t, $J = 7.2$ Hz, 3H), 2.88–2.91 (m, 2H), 2.93–2.96 (m, 2H), 3.35 (t, $J = 7.2$ Hz, 2H), 3.69 (s, 2H), 3.81 (s, 2H), 6.38 (s, 1H), 7.05 (s, 1H). ^{13}C NMR (DMSO) *δ*: 14.8, 23.7, 35.2, 57.9, 104.9, 111.6, 111.8, 112.8, 115.8, 131.3, 154.6, 155.8, 158.0, 161.4, 169.8. MS (ESI): *m/z* 352–353.9 $[M+H]^+$.

5.1.6. 5-(2,4-Diacetoxy-5-isopropylbenzoyl)-N-ethyl-4,5,6,7-tetrahydroisoxazole[4,5-c]pyridine-3-carboxamide (**23**)

To a solution of **19** (50 mg, 0.13 mmol) in DCM (10 mL) was added Ac_2O (54 mg, 50 μL, 0.53 mmol) and catalytic amount of pyridine. The reaction mixture was stirred for 4 h at room temperature, water (10 mL) and DCM (10 mL) were then added. The organic phase was separated, dried and the solvent evaporated under reduced pressure to give a residue which was purified by flash chromatography (DCM/MeOH 98/2) Yield 73%. ^{1}H NMR (400 MHz, CD_3OD): δ 1.10–1.21 (m, 9H), 2.10 (s, 3H), 2.32 (s, 3H), 2.90–3.08 (m, 5H), 3.24–3.35 (m, 2H), 3.75 (s, 2H), 4.54 (s, 2H), 7.03 (s, 1H), 7.33 (s, 1H), 8.23 (br, 1H). MS (ESI): *m/z* 458.1 $[M+H]^+$.

5.1.7. 5-(2,4-Bis-(benzyloxy)-5-isopropylbenzoyl)-4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridine-3-carboxylic acid (**24**)

Compound **12** (0.99 mmol, 373 mg), EDC (0.99 mmol, 175 μL), HOBt (0.99 mmol, 134 mg) and DIPEA (1.98 mmol, 344 μL) were added to a solution of 2,4-bis-(benzyloxy)-5- isopropylbenzoic acid [37] (0.82 mmol, 191 mg) in 5 mL of DMF. The reaction was stirred overnight at room temperature; then it was poured into water and extracted with AcOEt. The organic phase was washed with water and brine, then dried and evaporated. The crude product was purified by flash chromatography (light petroleum/AcOEt 7/3) to give the intermediate ethyl 5-(2,4-bis(benzyloxy)-5-isopropylbenzoyl)-4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridine-3-carboxylate. Yield 85%. ^{1}H NMR (200 MHz, DMSO, 120 °C): δ 1.17 (d, $J = 7.0$ Hz, 6H), 1.28 (t, $J = 7.0$ Hz, 3H), 2.79–2.85 (m, 2H), 3.26 (ept, $J = 7.0$ Hz, 1H), 3.72–3.77 (m, 2H), 4.35 (q, $J = 7.0$ Hz, 2H), 4.49–4.53 (m, 2H), 5.09 (s, 2H), 5.18 (s, 2H), 6.89 (s, 1H), 7.04 (s, 1H), 7.28–7.48 (m, 10H). MS (ESI): *m/z* 555.3 $[M+H]^+$.

The obtained ester (0.7 mmol, 386 mg) was dissolved in a solution of $LiOH \cdot H_2O$ (0.58 mmol, 38 mg) in 15 mL of THF/H_2O 3:2. The reaction mixture was stirred until complete conversion of the starting material to the corresponding carboxylic salt derivative. THF was evaporated and the aqueous phase was acidified with 1 N HCl and extracted with AcOEt (20 mL). The organic phase was dried and evaporated to give the desired acid, which was used without further purification.

5.1.7.1. 5-(2,4-Dihydroxy-5-isopropyl-benzoyl)-4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridine-3-carboxylic acid (2-morpholin-4-yl-ethyl)-amide (**25**).

To a solution of acid **24** (0.17 mmol, 90 mg) in 5 mL of DCM, 4-(2-aminoethyl)-morpholine (0.25 mmol, 32 μL), DIPEA (0.5 mmol, 86 μL), and PyBOP (0.2 mmol, 106 mg) were added. The reaction was stirred overnight at room temperature; the DCM was removed in vacuo and water was added. The mixture was then extracted with AcOEt (3 × 15 mL). The organic phase was dried over Na_2SO_4 to obtain the protected product as white solid. MS (ESI): *m/z* 661.3 $[M+Na]^+$.

The desired final compound was obtained by deprotection with BCl_3 1 M in DCM as previously described for compound **21**. The crude product was purified by flash chromatography (DCM/methanol from 98/2 to 96/4) to give 47 mg of the title compound. Yield 62%. ^{1}H NMR (300 MHz, DMSO) δ 1.08 (d, $J = 7.0$ Hz, 6H), 2.44–2.35 (m, 6H), 2.87 (m, 2H), 3.05 (m, 1H), 3.30 (m, 2H), 3.53 (t, $J = 4.5$ Hz, 4H), 3.71 (bs, 2H), 4.52 (bs, 2H), 6.36 (s, 1H), 6.87 (s, 1H), 8.58 (t, $J = 5.6$ Hz, 1H), 9.55 (s, 2H). MS (ESI): *m/z* 459.4 $[M+H]^+$.

5.1.7.2. 5-(2,4-Dihydroxy-5-isopropyl-benzoyl)-4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridine-3-carboxylic acid cyclopentylamide (**26**).

To a solution of acid **24** (0.17 mmol, 90 mg) in 5 mL of DCM, cyclopentylamine (0.25 mmol, 25 μL), DIPEA (0.25 mmol, 43 μL), and PyBOP (0.2 mmol, 106 mg) were added. The reaction was stirred for 6 h at room temperature; then it was poured into water and extracted with AcOEt (3 × 15 mL). The organic phase was dried over Na_2SO_4 and evaporated. The crude product was purified by flash chromatography (hexane/AcOEt 70/30) to give 84 mg of the protected title compound. Yield 82%. MS (ESI): *m/z* 616.2 $[M+Na]^+$.

The desired final compound **26** was obtained by deprotection with BCl_3 1 M in DCM as previously described for compound **21**. Yield 67%. ^{1}H NMR (300 MHz, DMSO) δ 1.08 (d, $J = 6.9$ Hz, 6H), 1.9–1.4 (m, 8H), 2.87 (m, 2H), 3.05 (m, 1H), 3.71 (bs, 2H), 4.15 (m, 1H), 4.51 (bs, 2H), 6.36 (s, 1H), 6.87 (s, 1H), 8.65 (d, $J = 7.5$ Hz, 1H), 9.54 (s, 2H). MS (ESI): *m/z* 414.3 $[M+H]^+$.

5.1.7.3. 5-(2,4-Dihydroxy-5-isopropyl-benzoyl)-4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridine-3-carboxylic acid (2-chloro-ethyl)-amide (**27**).

2-Chloroethylamine hydrochloride (0.25 mmol, 29 mg),

DIPEA (0.5 mmol, 86 μL), and PyBOP (0.2 mmol, 106 mg) were added to a solution of acid **24** (0.17 mmol, 90 mg) in 5 mL of DCM. The reaction was stirred overnight at room temperature; the DCM was removed in vacuo and water was added (10 mL). The mixture was then extracted with AcOEt (3 × 15 mL). The organic phase was dried over Na_2SO_4 and evaporated. The crude product was purified by flash chromatography (hexane/AcOEt 7/3) to give 90 mg of protected product as white solid. Yield 88%. ESI-MS m/z = 610.3/612.3 $[M+Na]^+$. The desired compound was obtained by deprotection with BCl_3 1 M in DCM as previously described for compound **21**. The crude product was purified by flash chromatography (DCM/methanol 98/2) to give 40 mg of the title compound. Yield 65%. ^1H NMR (300 MHz, DMSO) δ 1.08 (d, J = 6.9 Hz, 6H), 2.88 (m, 2H), 3.05 (m, 1H), 3.54 (m, 2H), 3.69 (bm, 4H), 4.53 (bs, 2H), 6.36 (s, 1H), 6.87 (s, 1H), 8.92 (t, J = 5.5 Hz, 1H), 9.54 (s, 2H). MS (ESI): m/z 408.32/410.34 $[M+H]^+$.

*5.1.7.4. Methyl 7-((5-(2,4-dihydroxy-5-isopropyl-benzoyl)-6,7-dihydro-4H-isoxazolo[4,5-c]pyridine-3-carboxamido)-heptanoate (**28**).* Methyl 7-aminoheptanoate HCl (0.20 mmol, 39 mg), DIPEA (0.40 mmol, 70 μL) and PyBOP (0.20 mmol, 106 mg) were added to a solution of acid **24** (0.17 mmol, 92 mg) in 5 mL of DCM. The reaction mixture was stirred at room temperature until complete conversion of starting material to amide (4 h, TLC check with eluent DCM/MeOH 96/4) then diluted with DCM (15 mL) and washed with H_2O (10 mL). The organic phase was dried over Na_2SO_4 and concentrated at reduced pressure. The crude product was purified by flash chromatography (DCM/MeOH = 98/2) to give methyl 7-((5-(2,4-dibenzyloxy-5-isopropyl-benzoyl)-6,7-dihydro-4H-isoxazolo[4,5-c]pyridine-3-carboxamido)-heptanoate (98 mg, Yield 88%).^1H NMR (300 MHz, DMSO, at 80 °C) δ 1.16 (d, J = 6.9 Hz, 6H), 1.32 (m, 4H), 1.55 (m, 4H), 2.27 (t, J = 7.3 Hz, 2H), 2.78 (m, 2H), 3.24 (m, 3H), 3.58 (s, 3H), 3.72 (bs, 2H), 4.51 (bs, 2H), 5.07 (s, 2H), 5.16 (s, 2H), 6.87 (s, 1H), 7.03 (s, 1H), 7.28 (s, 5H), 7.42 (m, 5H), 8.11 (bs, 1H). MS (ESI): m/z 690.2 $[M+Na]^+$.

Compound **28** was obtained by deprotection with BCl_3 1 M in DCM as previously described for compound **21**. The crude product was purified by flash chromatography (DCM/MeOH 97/3) to give 40 mg of the desired product (Yield 58%). ^1H NMR (300 MHz, DMSO) δ 1.08 (d, J = 6.9 Hz, 6H), 1.24 (m, 4H), 1.47 (m, 4H), 2.26 (t, J = 7.4 Hz, 2H), 2.87 (m, 2H), 3.04 (m, 1H), 3.17 (m, 2H), 3.55 (s, 3H), 3.71 (bs, 2H), 4.51 (bs, 2H), 6.36 (s, 1H), 6.86 (s, 1H), 8.72 (t, J = 5.8 Hz, 1H), 9.57 (bs, 2H). MS (ESI): m/z 488.1 $[M + H]^+$, 510.1 $[M + Na]^+$, 486.1 $[M-H]^-$.

*5.1.7.5. 5-(2,4-Dihydroxy-5-isopropyl-benzoyl)-N-[7-(hydroxyamino)-7-oxo-heptyl]-6,7-dihydro-4H-isoxazolo[4,5-c]pyridine-3-carboxamide (**29**).* Hydroxylamine (50% aqueous solution, 0.61 mmol, 41 μL) and NaOH 1 M (0.41 mmol, 410 μL) were added to a stirred solution of **28** (20 mg) in MeOH (410 μL, 0.1 M) at 0 °C. The reaction mixture was warmed up to room temperature and after 30 min TLC analysis showed complete conversion of starting material. The solution was cooled to 0 °C, neutralized with HCl 1 M (310 μL) and then extracted with AcOEt. The organic phase was washed with water and with brine, dried over Na_2SO_4 and concentrated under reduced pressure to give **29** (20 mg, Yield 99%). ^1H NMR (300 MHz, DMSO) δ 1.08 (d, J = 7.0 Hz, 6H), 1.22 (m, 4H) 1.45 (m, 4H), 1.91 (t, J = 7.3 Hz, 2H), 2.87 (m, 2H), 3.04 (m, 1H), 3.17 (m, 2H), 3.71 (bs, 2H), 4.51 (bs, 2H), 6.36 (s, 1H), 6.86 (s, 1H), 8.70 (bs, 1H), 8.72 (t, J = 5.6 Hz, 1H), 9.55 (bs, 2H), 10.35 (bs, 1H). MS (ESI): m/z 489.1 $[M+H]^+$, 511.2 $[M+Na]^+$, 487.1 $[M-H]^-$.

5.2. Biology

5.2.1. Cellular sensitivity to drugs

In non-small-cell lung carcinoma cells (NCI-H460) cellular sensitivity to drugs was evaluated by growth-inhibition assay after 72 h of drug exposure. Cells in the logarithmic phase of growth were seeded into 96-well plates, and 24 h after seeding, the drug was added to the medium. Cell survival was evaluated after 72 h of drug exposure by the sulforhodamine B test. IC_{50} was calculated by the ALLFIT program and was defined as the drug concentration causing a 50% reduction of cell number compared to that of untreated control cells.

5.2.2. Binding on Hsp90 by a fluorescence polarization assay [38]

GM-FITC, supplied by Invivogen (catalog no. 06C23-MT, CA, U.S.) was previously dissolved in DMSO to obtain 10 mM stock solutions and kept at −20 °C until use. Hsp90, purchased from Stressgen (catalog no. SPP-776, Victoria, BC, Canada), was previously dissolved in assay buffer (HFB) to form 2.2 μM stock solutions and kept at −80 °C until use. The compounds were previously dissolved in DMSO to obtain stock solutions and kept at −20 °C. On the day of the experiment, various concentration solutions were prepared by serial dilutions in assay buffer (HFB) containing 20 mM HEPES (K), pH 7.3, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Na_2MoO_4, and 0.01% NP40. Before each use, 0.1 mg/mL bovine γ globulin and 2 mM DTT were freshly added. Fluorescence polarization (FP) was performed in Opti-Plate-96F well plates (Perkin–Elmer, Zaventem, Belgium) using a plate reader (Wallac Envision 2101 multilabel reader, Perkin–Elmer, Zaventem, Belgium). To evaluate the binding affinity of the molecules, an amount of 50 μL of the GM-FTC solution (5 nM) was added to 30 nM Hsp90 in the presence of 5 μL of the test compounds at increasing concentrations. The plates were shaken at 4 °C for 4 h, and the FP values in mP (millipolarization units) were recorded. The IC_{50} values were calculated as the inhibitor concentration that displaced 50% of the tracer, each data point being the result of the average of triplicate wells, and were determined from a plot using nonlinear least-squares analysis. Curve fitting was performed using Prism GraphPad software program (GraphPad Software, Inc., San Diego, CA, U.S.)

5.2.3. Growth inhibition and apoptosis in K562 cells

5.2.3.1. Cytotoxicity assay. To evaluate the number of live and dead cells, cells were stained with trypan blue and counted on a hemocytometer. Cells which showed trypan blue uptake were interpreted as nonviable. To determine the growth inhibitory activity of the drugs tested, 2×10^5 cells were plated into 25-mm wells (Costar, Cambridge, U.K.) in 1 mL of complete medium and treated with different concentrations of each drug. After 48 h of incubation, the number of viable cells was determined and expressed as percent of control proliferation.

5.2.3.2. Flow cytometric analysis per cell cycle and apoptosis. The cells were washed once in ice-cold PBS and resuspended at 1×10^6 cells/mL in a hypotonic fluorochrome solution containing propidium iodide (Sigma), 50 g/mL in 0.1% sodium citrate plus 0.03% (v/v) Nonidet P-40 (Sigma). After 30 min of incubation in this solution, the samples were filtered through nylon cloth, and their fluorescence was analyzed as single-parameter frequency histograms using a FACSort (Becton Dickinson, Mountain View, CA). The distribution of cells in the cell cycle was determined using the ModFit LT program (Verity Software House, Inc.). Apoptosis was determined by evaluating the percentage of hypoploid nuclei accumulated in the sub-G_0–G_1 peak after labeling with propidium iodide.

5.2.3.3. Histone deacetylase profiling. HDAC profiling was performed by Reaction Biology Corp. (Malvern, PA) against 10 isolated isoforms of human HDAC (HDAC1–8, 10–11) in the presence of the fluorogenic tetrapeptide RHKKAc (p53 residues 379–382) as the substrate (10 μM). Isolated human HDACs were obtained by standard purification, with the exception of HDAC3, which was isolated in complex with NCOR2 and used as such. SAHA was used as reference compounds. Each compound was dissolved in DMSO (10 μM), and sequentially diluted solutions were used for testing. IC_{50} values were calculated from the resulting sigmoidal dose–response inhibition slopes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.01.056.

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