Bioorganic & Medicinal Chemistry xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Discovery of NMS-E973 as novel, selective and potent inhibitor of heat shock protein 90 (Hsp90)

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ARTICLE INFO

Article history:
Received 31 May 2013
Revised 4 September 2013
Accepted 6 September 2013
Available online xxxx

Keywords:
Fragment based drug discovery
Tumor cell proliferation inhibition
Anti-cancer agents

ABSTRACT

Novel small molecule inhibitors of heat shock protein 90 (Hsp90) were discovered with the help of a fragment based drug discovery approach (FBDD) and subsequent optimization with a combination of structure guided design, parallel synthesis and application of medicinal chemistry principles. These efforts led to the identification of compound **18** (NMS-E973), which displayed significant efficacy in a human ovarian A2780 xenograft tumor model, with a mechanism of action confirmed in vivo by typical modulation of known Hsp90 client proteins, and with a favorable pharmacokinetic and safety profile.

1. Introduction

Heat shock protein 90 (Hsp90) is a molecular chaperone, which plays a key role in the conformational maturation, stability and function of several oncogenic proteins that are involved in signal transduction pathways, whose dysfunctional activation have been collectively described as constituting the hallmark traits of cancer.[1,2] Discovery and characterization of natural inhibitors of Hsp90 function such as geldanamycin[3] and radicicol[4] have attracted interest on Hsp90 as a therapeutic target for anticancer drugs. It was shown that geldanamycin inhibits Hsp90 functions by binding to an ATP binding pocket in the N-terminal domain of the protein. The subsequent loss of its chaperone function induces the degradation of diverse client proteins. After the identification of the early Hsp90 inhibitors, in particular 17-AAG[5] and other semi-synthetic ansamycin derivatives such as retaspimycin[6] a new group of fully synthetic second generation inhibitors was developed with improved preclinical efficacy and "drug-likeness" including the intravenous drugs NVP-AUY922,[7] AT-13387,[8] ganetespib (STA-9090),[9] KW-2478,[10] and the oral drugs DEBIO-0932 (CUDC-305),[11] NVP-HSP990,[12] SNX-5422,[13] and PU-H71,[14] which have progressed into clinical development.

As part of our strategy for the identification of novel chemotypes for the development of Hsp90 inhibitors we relied on fragment based drug discovery (FBDD), an approach receiving increased recognition for its performance in delivering efficient ligands and focused optimization pathways.[15] We screened our corporate fragment library characterized by low molecular weight compounds (<300 Da) against the N-terminal domain of human Hsp90α using a FAXS (Fluorine chemical shift Anisotropy and eXchange for Screening) NMR displacement assay.[16] The screening led to the identification of fragment **1** (Fig. 1), which showed a Kd of 0.6 μM in the FAXS competitive binding assay. On the basis of these results, fragment **1** was selected as starting point for further explorations. To facilitate the chemical expansion we determined the crystal structure of **1** bound to the ATPase N-terminal domain of Hsp90 (Fig. 1). The resorcinol moiety of **1** occupies the adenosine pocket and displays a binding mode already described for other known Hsp90 inhibitors:[7,8] one of the two phenolic hydroxyl groups makes key interactions with Asp93 and with a conserved water molecule, whereas the other hydroxyl substituent is involved in a network of water mediated interactions with the nearby Ser52 and Leu48 residues. The dichlorophenyl group is stacked between the side chain of Leu107 and Phe138 and fills a hydrophobic pocket adjacent to the adenosine binding site, created by the rearrangement of residues 103–111, as previously reported also for other Hsp90 inhibitors.[11,14,17–19] Based on modeling studies, our exploration and optimization strategy was focused on expansion of the initial hit within the ATP binding site, by adding groups pointing to the solvent region, and on improvement of its fitting into the induced hydrophobic pocket, through the modification of the substitution pattern of the phenyl ring.

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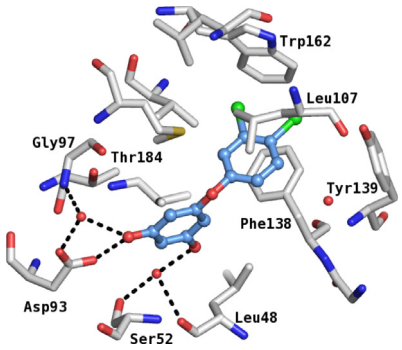
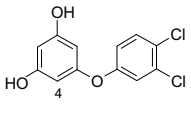
http://dx.doi.org/10.1016/j.bmc.2013.09.018





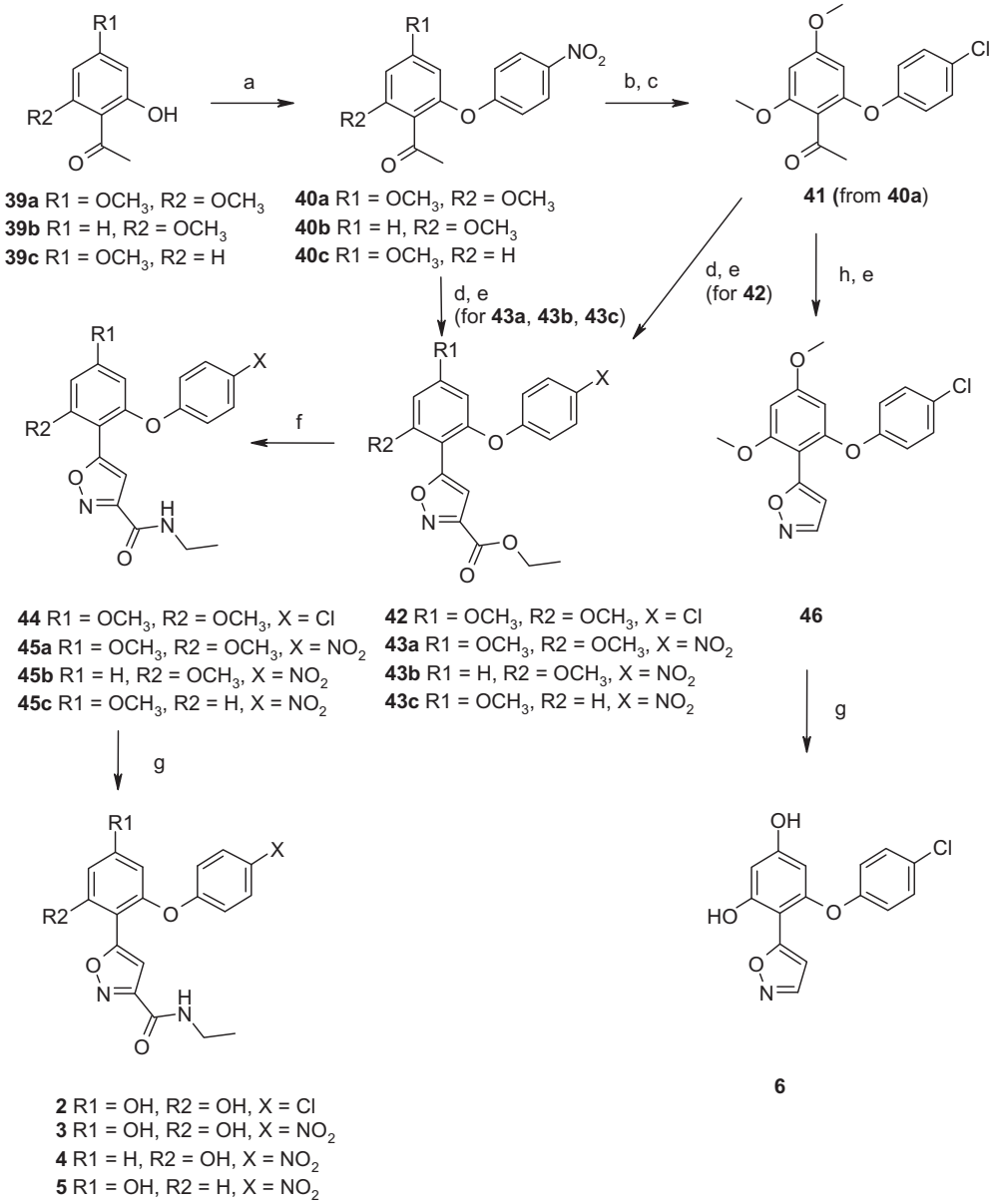
1

Figure 1. Compound **1** bound to the active site of Hsp90. Compound **1** is shown with blue carbon atoms while protein is colored in grey. Ordered water molecules are shown as red spheres and hydrogen bonds as dashed line.

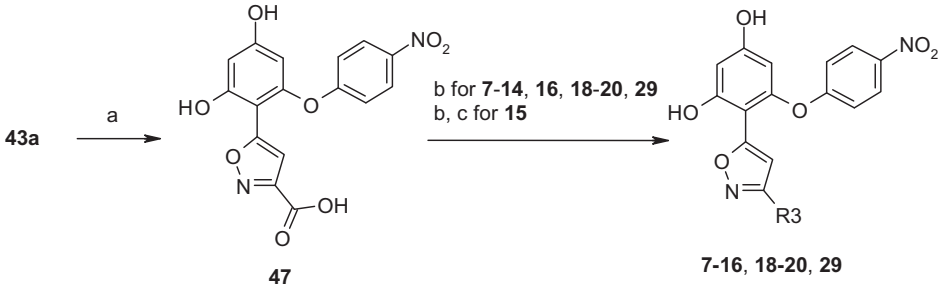
Comparison of **1** to crystal structures of other known Hsp90 inhibitors, in particular of VER-49009[20] and NVP-AUY922,[7] suggested to focus initially on the 4-position of the resorcinol ring of **1**, by adding a moiety like an isoxazole 3-carboxamide pointing to the solvent region, with the aim of increasing the binding affinity through additional interactions with the protein.

2. Chemistry

Compounds **2**–**38** were synthesized[21] according to Schemes 1–4. Reaction of commercially available **39a–c** with 1-fluoro-4-nitrobenzene in DMSO in presence of potassium carbonate afforded **40a–c**. Reduction of **40a** furnished the amino derivative, which was converted into the chloro analogue **41** via its diazonium salt. Treatment of **40a–c** and **41** with diethyl oxalate in the presence of lithium bis(trimethylsilyl)amide in THF and final cyclization with hydroxylamine hydrochloride in ethanol afforded isoxazole



Scheme 1. Reagents and conditions: (a) 1-Fluoro-4- nitrobenzene, K_2CO_3, DMSO, 110 °C, 2 h; (b) 37% HCl, Pd/C, H_2, MeOH/EtOAc/DCM (1:1:1), 40 psi, 8 h; (c) $CuCl_2$, *t*-BuONO, acetonitrile, 65 °C, 2 h; (d) 1 M Lithium bis(trimethylsilyl)amide in THF, −50 °C, then $(COOEt)_2$, rt, 2 h; (e) $NH_4OH.HCl$, EtOH, reflux, 2 h; (f) 2 N $EtNH_2$ in THF, 100 °C, 5 h; (g) 1 M BBr_3 in DCM, rt, 120 h; (h) 1 M Lithium bis(trimethylsilyl)amide in THF, −50 °C, then HCOOEt, rt, 2 h.

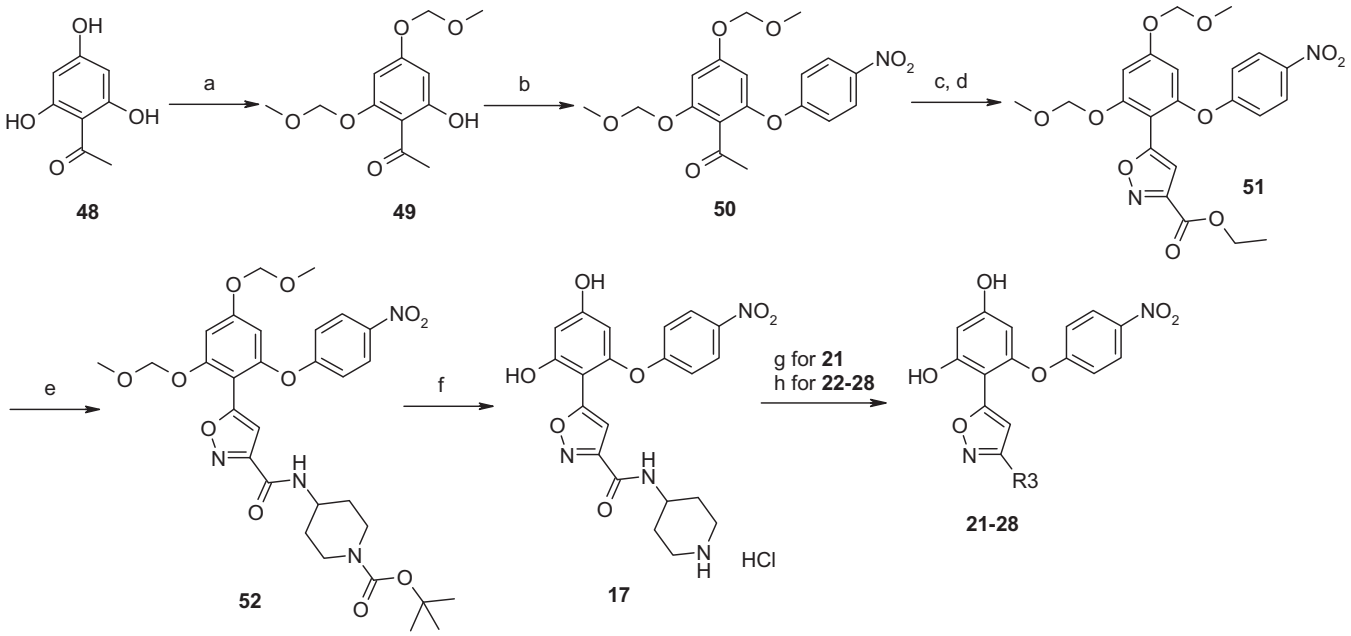


Scheme 2. Reagents and conditions: (a) 1 M BBr_3 in DCM, rt, 120 h; (b) primary or secondary amines, DIPEA, TBTU, DMA, rt, overnight; (c) 50% CF_3COOH, DCM, rt, 1 h.

derivatives **42** and **43a–c**. Conversion of the ester function to ethylamides **44** and **45a–c** was obtained in one step by reaction with ethylamine in THF in a stopped flask at 100 °C. Finally, treatment with BBr_3 furnished the target compounds **2–5** (Scheme 1). Reaction of **41** with ethyl formate, in the presence of lithium bis(trimethylsilyl)amide in THF, followed by cyclization with hydroxylamine hydrochloride in ethanol afforded **46**, which was finally converted to compound **6** by treatment with BBr_3. Preparation of amides **7–16**, **18–20** and **29** was accomplished according to Scheme 2, starting from carboxylic ester **43a**. BBr_3 mediated removal of hydroxyl protecting groups and, at the same time, saponification of the carboxylic ester afforded **47**, which was reacted with suitable amines under usual coupling condition to give **7–14**, **16**, **18–20** and **29**, while **15** was obtained after nitrogen deprotection of the piperidine ring. A better look at the previous synthetic procedure led to focus our attention on hydroxyl protecting group removal. In fact, despite the use of an excess of BBr_3, after five days the reaction was not completed. Furthermore, recovery of pure material required extensive purification. The synthetic pathway was ameliorated (Scheme 3) using a different protecting group. The in situ methoxymethylation[22] of **48** afforded **49**,[23] which was then reacted with 1-fluoro-4-nitrobenzene at 55 °C. Condensation of **50** with diethyloxalate was carried out using sodium *tert*-butoxide at −10 °C, the raw intermediate was cyclized by treatment with hydroxylamine hydrochloride at room temperature to isoxazole **51**, which was converted to **52** by aminolysis with *tert*-butyl 4-aminopiperidine-1-carboxylate. Removal of the protecting groups under acidic conditions afforded **17**. Alkylation of **17** with 1-bromo-4,4,4-trifluorobutane afforded **21**, while **22–28** were prepared by reductive amination. Preparation of **30–38** was accomplished according to Scheme 4. Aminolysis of **51** with 1-methyl-piperidin-4-ylamine furnished **53.** Reduction of the nitro group of **53** afforded **54**, which was then converted to **30–31** and **33–34** by alkylation of the piperidine nitrogen under reductive amination condition followed by protecting groups removal. Compound **32** was prepared by reaction of **54** with 2,5-dimethoxytetrahydrofuran, followed by protecting groups removal. Finally, reduction of the nitro group of **52** followed by alkylation of the amino group with formaldehyde under reductive amination condition furnished its dimethylamino derivative **55**, which was converted into **35** by protecting groups removal. Alkylation of **35** with 1-bromo-4,4,4-trifluorobutane afforded **36**, while **37–38** were prepared by reductive amination.

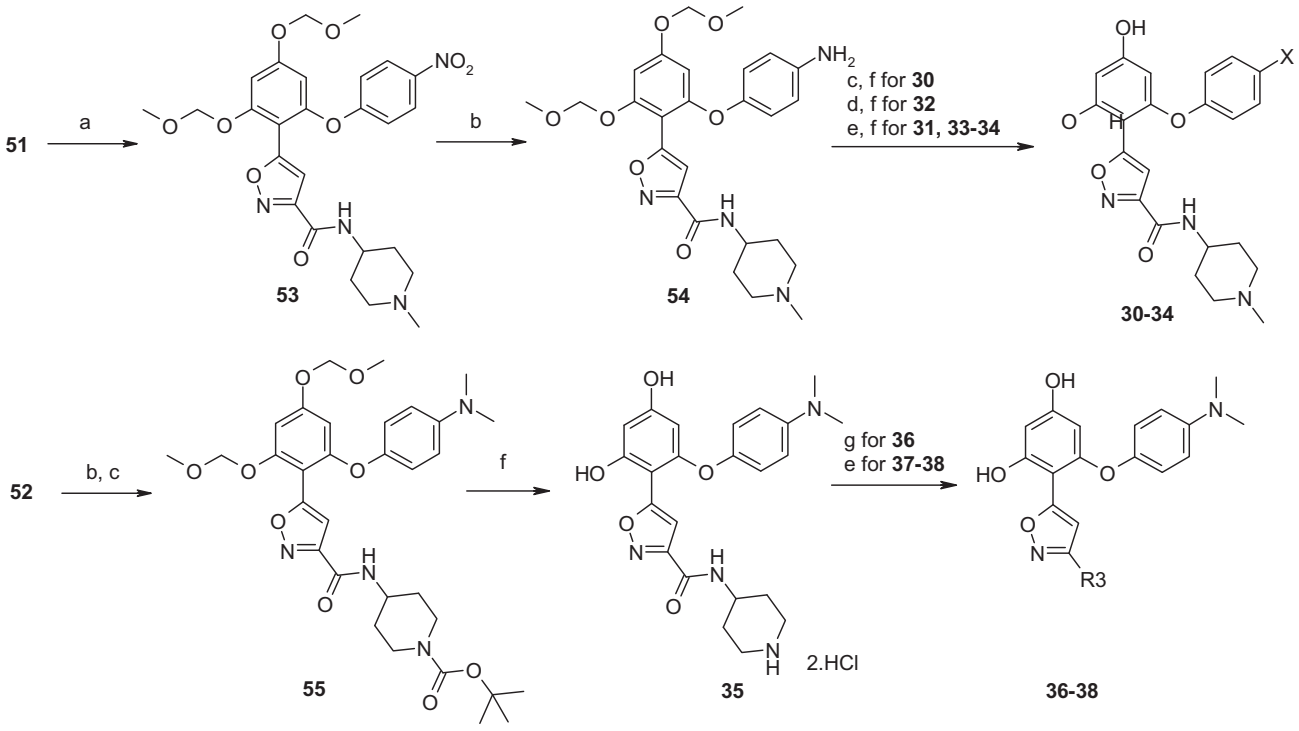
3. Results and discussion

Table 1 summarizes the SAR of the first set of compounds **2–6**. Binding to Hsp90 was measured by fluorescence polarization (FP) assay and inhibition of cell growth proliferation was determined on A2780 tumor cell line. Due to interference with the fluorescence



Scheme 3. Reagents and conditions: (a) Dimethoxymethane, $ZnBr_2$, DCM, AcCl, then **48**, DIPEA, DCM, 5 °C, 1 h, then rt, on; (b) 1-fluoro-4-nitrobenzene, K_2CO_3, DMSO, 55 °C, 6 h; (c) *t*-BuONa, $(COOEt)_2$, THF, −10 °C, 1 h, then rt, 2 h; (d) $NH_2OH{\cdot}HCl$, AcONa, EtOH, rt, 2 h, then 6 N HCl, rt, 24 h; (e) *t*-butyl 4-aminopiperidine-1-carboxylate, 80 °C, 24 h; (f) 4 N HCl in dioxane, EtOH, rt, 5 h; (g) 1-bromo-4,4,4-trifluorobutane, DIPEA, DMF, rt, overnight; (h) aldehydes or ketones, $(CH_3)_4N(OAc)_3BH$, DMF, rt, overnight.

Please cite this article in press as: Brasca, M. G.; et al. *Bioorg. Med. Chem.* (2013), http://dx.doi.org/10.1016/j.bmc.2013.09.018

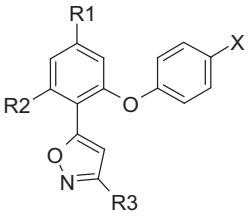


Scheme 4. Reagents and conditions: (a) 1-Methyl-piperidin-4-ylamine, 80 °C, 9 h; (b) Zn, NH_4Cl, dioxane, H_2O, 100 °C, 3 h; (c) 37% HCHO, $(CH_3)_4N(OAc)_3BH$, AcOH, DCM, DMF, rt, 1 h; (d) 2,5-dimethoxytetrahydrofuran, 2.5 M H_2SO_4, $NaBH_4$, THF, MeOH, rt, overnight; (e) ketones, $(CH_3)_4N(OAc)_3BH$, AcOH, DMF, rt, overnight; (f) 4 N HCl in dioxane, EtOH, rt, overnight; (g) 1-bromo-4,4,4-trifluorobutane, DIPEA, DMF, rt, overnight.

signal the FP data for fragment hit **1** could not be generated, therefore its binding affinity was measured by the FAXS competitive binding assay and the Kd resulted to be 0.6 μM. Gratifyingly, compounds **2** and **3**, where an isoxazole 3-carboxamide moiety was added on the 4-position of the resorcinol ring of **1**, showed improved binding affinity to Hsp90. A deeper investigation from a biological and chemical point of view was then pursued. It was observed that the replacement of chloro (**2**) with nitro (**3**) maintained the affinity to Hsp90, while the activity in cells increased about fourfold. Removal of one of the two hydroxyl moieties (**4**–**5**) led to a drop in enzyme affinity and potency in cells, confirming the crucial role of the concurrent presence of the two hydroxyl groups in the molecule. Removal of ethylcarboxamide (**6**) caused approximately a fourfold decrease of potency in the biochemical assay with respect to **2**, while a more drastic reduction of the antiproliferative activity was observed.

The crystal structure of **3** (Fig. 2) shows that the resorcinol moiety maintains the key interactions with Asp93 and the conserved waters. The carboxamide function, expanding towards the solvent area, makes additional hydrogen bond interactions with the carbonyl backbone of Gly97 and with the nitrogen side chain of Lys58. Interestingly, the *p*-nitrophenyl group of **3** does not occupy anymore the hydrophobic pocket induced by compound **1**. The aromatic group rotates of about 48 degrees with respect to the dichlo-

Table 1
Preliminary exploration



Compd	R1	R2	R3	X	Hsp90 DC_{50}[a] (μM)	A2780 IC_{50}[a] (μM)
2	OH	OH	CONHEt	Cl	0.052	0.300
3	OH	OH	CONHEt	NO_2	0.048	0.080
4	H	OH	CONHEt	NO_2	1.920	>10
5	OH	H	CONHEt	NO_2	>10	>10
6	OH	OH	H	Cl	0.210	>10

[a] Values are means of three experiments.

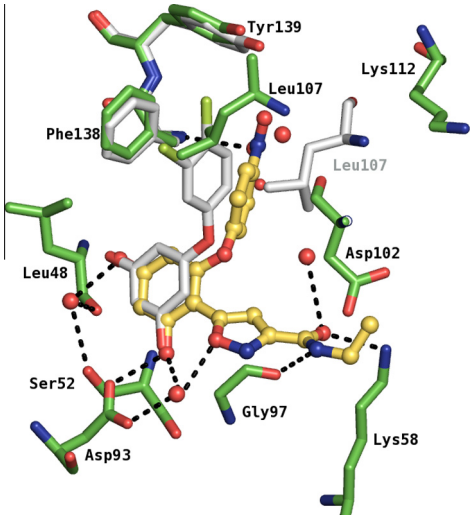
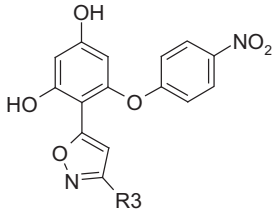
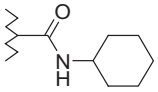
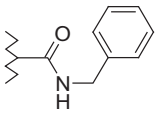
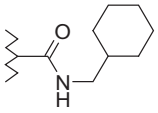
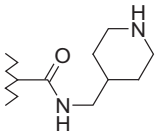
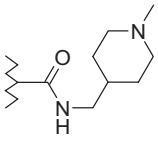
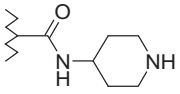
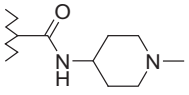
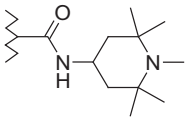
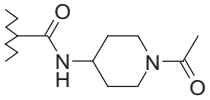
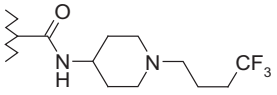
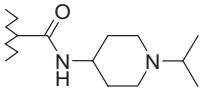
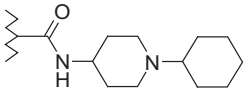


Figure 2. Overlay of the crystal structures of bound compounds **3** and **1**. Compound **3** is shown with yellow carbon atoms, while the protein is colored in green. Carbon atoms of **1** and the protein residues interacting with it are shown in grey. Ordered water molecules are shown as red spheres and hydrogen bonds as dashed line.

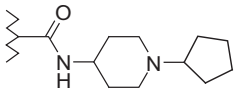
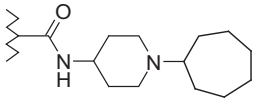
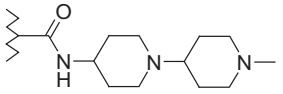
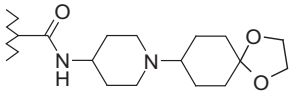
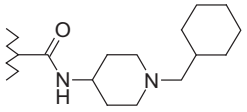
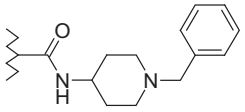
Table 2
R3 expansion



Compd	R3	Hsp90 DC_{50}[a] (μM)	HER2 degrad IC_{50} (μM)	A2780 IC_{50}[a] (μM)	Solubility pH 7 (μM)
3	$CONHCH_2CH_3$	0.048	0.114	0.080	65
7	$CON(CH_3)_2$	>10	ND	>10	145
8	$CONHCH_2CH_2F$	0.018	0.086	0.052	19
9	$CONHCH_2CH_2OCH_3$	0.021	ND	0.276	130
10	$CONHCH_2CH_2OH$	0.016	ND	0.294	131
11	$CONH(CH_2)_3N(CH_3)_2$	0.021	ND	0.192	84
12		0.658	ND	1.946	18
13		2.713	ND	7.271	14
14		>10	ND	>10	<1
15		0.025	ND	>10	144
16		0.033	ND	7.188	125
17		0.017	ND	0.362	155
18		0.010	0.110	0.069	179
19		0.013	ND	0.396	104
20		0.073	ND	3.404	ND
21		0.022	0.071	0.060	40
22		0.019	0.277	0.052	143
23		0.020	0.071	0.050	61

(*continued on next page*)

Table 2 (*continued*)

Compd	R3	Hsp90 DC_{50}[a] (μM)	HER2 degrad IC_{50} (μM)	A2780 IC_{50}[a] (μM)	Solubility pH 7 (μM)
24		0.054	0.210	0.029	75
25		0.048	0.130	0.029	58
26		0.012	ND	0.368	138
27		0.037	0.088	0.041	87
28		0.046	0.032	0.028	25
29		0.035	0.094	0.013	9

ND = not determined.
[a] Values are means of three experiments.

rophenyl moiety of **1** and it is situated near Leu107 and Phe138 residues in a solvent exposed site. The nitro group is located in the ADP α-phosphate site and makes a hydrogen bond to the Phe138 backbone nitrogen. This structural information led us to slightly modify our chemical expansion strategy, as described later in this paper. The initial plan to introduce bulkier groups on the aromatic ring, which could better fit into the hydrophobic pocket, was halted.

Based on the structural analysis of the carboxamide moiety of **3**, exposed to the solvent area, a larger series of residues linked to the isoxazole ring was investigated.

Data concerning activity and solubility in neutral buffer of analogues of **3** are summarized in Table 2. Furthermore, an assay measuring Her2 client protein degradation in BT-474 breast cancer cell line was also established to evaluate Hsp90 specific cellular effects of interesting compounds with an IC_{50} <100 nM on A2780 cells. Condensation of carboxylic acid **47** with different amines gave rise to a series of amides **7**–**29**, whose biochemical and cellular activity was sharply affected by minor modifications. The secondary nature of the amide was essential to binding, as deduced from the detrimental effect of a tertiary amide function on compound **7**, likely due to steric clash and loss of interaction with Gly97. Linear aliphatic amides bearing small hydrophilic residues (**8**–**11**) showed better binding affinity to Hsp90 with respect to **3**. Interesting potency in A2780 cell proliferation assay was observed for compound **8**, whereas **9**–**11** were less potent. Unfortunately, compound **8** displayed low solubility in neutral buffer. Bulkier and hydrophobic residues such as cyclohexyl, benzyl and cyclohexylmethyl (**12**–**14**) caused a marked decrease in solubility and in the protein affinity, probably due to the lack of interactions in an area rich of polar side chains. Improvement of solubility and affinity for Hsp90 with respect to **3** was achieved with compounds **15**–**18**, due to the presence of hydrophilic moieties. However, only **18** showed interesting inhibitory potency of cell growth proliferation of A2780 tumor cell line. Specific biological effects in BT-474 breast cancer cell line related to the inhibition of Hsp90 were determined for the most promising compounds **3**, **8** and **18**, with IC_{50} ranging from 86 nM for **8** to 114 nM for **3**. Co-crystal structure of **18** (Fig. 3) shows that the molecule maintains a similar binding mode to **3**. The resorcinol moiety makes the key interaction with Asp93 and the conserved water molecules while the carboxamide group makes hydrogen bond interactions with Gly97 and Lys58. Moreover, the basic piperidine nitrogen makes an additional salt interaction with the carboxylic residue of Asp102, thus increasing compound affinity. It is worth noting that the *p*-nitrophenyl group of **18** adopts a slightly

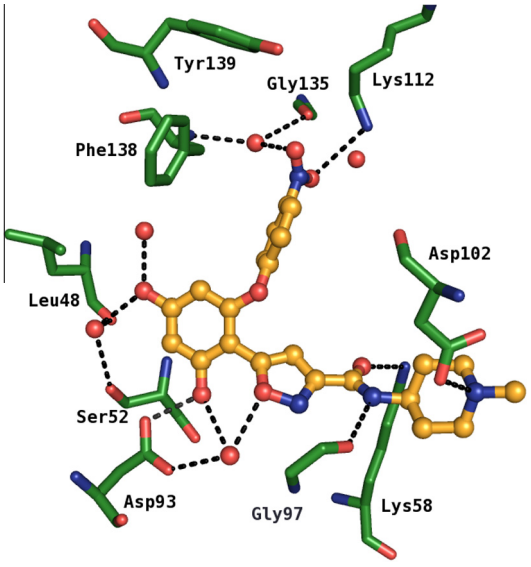
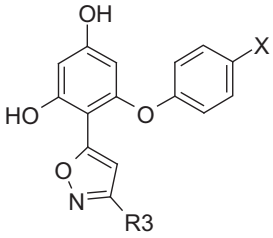
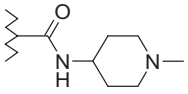
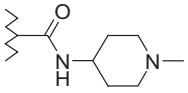
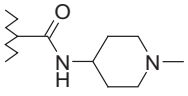
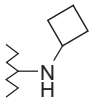
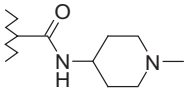
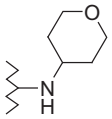
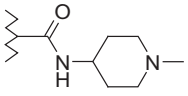
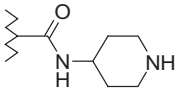
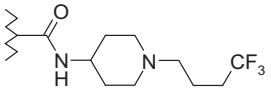
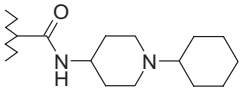
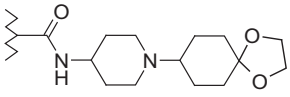


Figure 3. X-ray structure of the N-terminal ATP binding site of Hsp90 in complex with **18**. Compound **18** is shown with yellow carbon atoms, while the protein is colored in green. Ordered water molecules are shown as red spheres and hydrogen bonds as dashed lines.

Table 3
X and R3 expansion



Compd	X	R3	Hsp90 DC_{50}, μM[a]	HER2 degrad IC_{50} (μM)	A2780 IC_{50} (μM) [a]	Solubility pH 7 (μM)
30	$N(CH_3)_2$		0.016	0.033	0.037	161
31			0.013	0.065	0.074	158
32			0.037	0.107	0.088	119
33			0.061	ND	0.149	ND
34			0.104	ND	1.709	ND
35	$N(CH_3)_2$		0.025	ND	0.136	161
36	$N(CH_3)_2$		0.025	0.025	0.020	63
37	$N(CH_3)_2$		0.023	0.020	0.016	79
38	$N(CH_3)_2$		0.013	0.028	0.011	105

ND = not determined.
[a] Values are means of three experiments.

Table 4
Binding kinetic affinity and in vivo pharmacokinetic parameters in Balb nu/nu mice[b] of selected compounds

Compd	Hsp90 K_D[a] (nM)	PK data (iv), dose[c] 10 mg/kg			
		AUC_∞ (μM h)	CL (mL/min/kg)	V_{ss} (L/kg)	$t_{1/2}$ (h)
18	0.346 ± 0.001	9.26 ± 0.36	39.91 ± 1.70	5.83 ± 3.18	5.55 ± 1.07
30	0.120 ± 0.060	5.92 ± 0.26	62.29 ± 2.81	19.73 ± 1.61	5.55 ± 0.01
31	0.220 ± 0.069	4.80 ± 0.14	64.80 ± 1.97	18.37 ± 1.59	5.44 ± 0.53
38	0.057 ± 0.017	7.60 ± 0.69	29.35 ± 0.26	4.94 ± 0.42	5.46 ± 0.41

[a] Values are means of three experiments.
[b] $n = 3$ Animals per study.
[c] Dosed in 10% Tween 80 in 5% dextrose.

different conformation compared to the corresponding moiety of **3**, allowing a hydrogen bond of the nitro group with the side chain nitrogen of Lys112 and a water mediated interaction with Gly135 and Phe138. Comparison of the crystal structure of **18** with **3**[24] reveals a rearrangement of residues 109–124 so that Lys112 points toward the binding site. However, we can not exclude that the different conformation adopted by the *p*-nitrophenyl group is due to the change in protein conformation. Actually, the flexibility

of this protein region is known and was previously reported.[25] Compound **18** became the prototype of a series of analogues. Different structural motifs were chosen as a replacement of the methyl group of the piperidine ring. Modeling studies indicated that this part of the molecule protrudes into the solvent region allowing the accomodation of moieties of different size and lipophilicity.

Activity data concerning analogues of **18** (**19**–**29**) are summarized in Table 2. Sterically hindered 1,2,2,6,6-pentamethylpiperidin-4-yl **19** showed high binding affinity but lower antiproliferative activity on cells with respect to **18**, while acylation of the piperidine nitrogen (**20**) led to about a sevenfold decrease in the biochemical assay and to a more drastic reduction of the antiproliferative activity on the A2780 cell line. The lower affinity of **20** could be explained by loss of saline interaction between the piperidine nitrogen and the carboxylic residue of Asp102 of the protein, as seen for **18**. Replacement of the methyl group of the piperidine ring of **18** by linear, branched and cyclic aliphatic chains gave rise to compounds **21**–**23**, which maintained good affinity for Hsp90 and antiproliferative activity on cells. Her2 degradation in BT-474 cell line related to the inhibition of Hsp90 was observed for all the compounds, with an IC_{50} ranging from 71 nM (**21** and **23**) to 277 nM (**22**). Solubility in neutral buffer was moderate for **21** and **23**, and good for **22**. The noteworthy biological profile of **23** prompted the preparation of **24** and **25**, where the cyclohexyl moiety, located in the solvent region, was replaced by different size rings. A slight decrease in binding affinity and an improvement in antiproliferative activity on A2780 cells were observed with respect to **23**, while Her2 degradation IC_{50} was higher. Moreover, compounds **24** and **25** showed moderate solubility in neutral buffer. In order to improve solubility, the cyclohexyl moiety of **23** was replaced by the 1-methylpiperidin-4-yl group in **26**. However, despite its high affinity for Hsp90 and an improved solubility, this compound showed lower antiproliferative activity with respect to **23**. Compounds **27**–**29** were synthesized to further explore the solvent accessible region of Hsp90. These modifications led to a slight decrease in the binding affinity with respect to **18**, all of them exhibiting potent antiproliferative activity on A2780. Hsp90 dependent mechanism of action was confirmed in the Her2 degradation assay, however the solubility in neutral buffer, particularly for **28**–**29**, prevented further investigation.

With the aim of improving both affinity and physico-chemical properties we then explored the role of the nitro group of **3** by its replacement with different amino-substituted moieties (Table 3, **30**–**34**). Replacement of the nitro functionality with either dimethylamine or isopropylamine substituents (**30**–**31**) maintained affinity to Hsp90 and solubility, with an improvement of the cellular activity of **30** compared to **18**. Introduction of the pyrrolidine group (**32**) was tolerated, while cyclobutylamine and (tetrahydro-pyran-4-yl)-amine (**33**–**34**) caused a decrease in binding affinity and a more drastic reduction of the antiproliferative activity on the A2780 cell line, in particular for **34**. Her2 degradation assay confirmed the mechanism of action for compounds **30**–**32**, with an IC_{50} ranging from 33 nM for **30** to 107 nM for **32**.

The interesting results obtained with the dimethylamine derivative **30**, as far as potency and solubility were concerned, prompted the synthesis of carboxamides **35**–**38** (Table 3). The desmethyl analogue **35** showed an interesting Hsp90 affinity, but a lower antiproliferative effect than **30**. Insertion of the most interesting moieties seen in the previous expansion gave rise to compounds **36**–**38**, characterized by significant binding affinity, potent antiproliferative activity on cells, Hsp90 specific cellular effects on Her2 client protein and moderate solubility in neutral buffer.

Due to the high potency close to the sensitivity limit of the assay used, all the relevant compounds **18**, **30**, **31**, and **38**, selected on the basis of their affinity to Hsp90, antiproliferative activity, mechanism of action and preliminary solubility, were profiled by Surface Plasmon Resonance analysis, with an approach of kinetic titration as previously described,[26] in order to more accurately determine the K_D (Table 4). All compounds showed a K_D <1 nM. In addition, we tested for comparison in the same assay a reference Hsp90 inhibitor, SNX-2112,[13] and its measured K_D was 0.726 ± 0.015 nM.

No activity was observed when the selected compounds were tested against the biologically related ATPase Hsc70 as well as against a panel of 52 protein kinases (IC_{50} >10 nM).

In order to assess the potential of the most promising compounds **18**, **30**, **31**, and **38**, a preliminary pharmacokinetic investigation in healthy nude mice was performed. When administered intravenously (iv) at 10 mg/kg, all compounds showed good exposure levels and a half life >5 h (Table 4). High volume of distribution associated to a moderate systemic clearance was observed for **18** and **38**, indicating a good tissue distribution, while **30** and **31** showed an extremely high volume of distribution (V_{ss} >18 L).

Efficacy of **18** and **38** was evaluated in vivo in the human ovarian A2780 xenograft mouse model. As shown in Table 5, **18** was administered intravenously (iv) at 30 and 60 mg/kg for 10 consecutive days. Both schedules resulted in significant tumor growth inhibition (TGI = 53% and 74%, respectively), with a maximum body weight loss (BWL) of 11.5% observed only at higher dose, which rapidly recovered at the end of the treatment. Compound **38**, when given intravenously at 30 mg/kg for 10 consecutive days, showed higher tumor growth inhibition (TGI = 93%) with respect to **18**, but lower tolerability (BWL = 23.4%).

A PK/PD algorithm that links the dosing regimen to the A2780 tumor growth dynamics was generated,[27] suggesting a new administration schedule (60 mg/kg twice a day, 3 days on, 1 day off, 3 days on), which resulted in a better efficacy profile for compound **18**. With this optimized schedule, treatment with either 30 or 60 mg/kg of **18** resulted in high efficacy (TGI = 68% and 91%, respectively), associated with a moderate and reversible body weight loss (BWL = 4.5 and 7.7%, respectively). On the contrary, no improvement was achieved for compound **38** with this administration schedule. Treatment with either 10 or 15 mg/kg of **38** showed lower efficacy (TGI = 53% and 72%, respectively) in comparison to **18**, with no improvement in its safety profile.

We characterized the in vitro and in vivo effects and mechanism of action of compound **18**. Incubation of A2780 tumor cells with **18** induced degradation of AKT and inhibition of the AKT and MAPK pathways, as shown by the reduction of AKT and ERK phosphorylation, associated with the typical induction of Hsp70, a well-known feedback response to Hsp90 inhibition (Fig. 4). Analogous effects, although to a lesser extent, were observed in lysates from A2780 xenograft tumors isolated at different times after administration of 60 mg/kg, confirming that in vivo antitumor activity induced by **18** was Hsp90 dependent (Fig. 5).

Table 5
In vivo intravenous activity of compounds **18** and **38** on A2780 ovarian carcinoma in nude mice[a]

Compd	Treatment	Dose mg/kg	%TGI[b] (day)	%BWL[c] (day)
18	Die 1–10 day	30	53 (18)	2.4 (18)
18	Die 1–10 day	60	74 (18)	11.5 (18)
38	Die 1–10 day	30	93 (18)	23.4 (18)
18	1–3 bid × 2 cycles	30	68 (14)	4.5 (14)
18	1–3 bid × 2 cycles	60	91 (14)	7.7 (14)
38	1–3 bid × 2 cycles	10	53 (14)	12.3 (14)
38	1–3 bid × 2 cycles	15	72 (14)	19.1 (14)

[a] n = 7 Animals per study.
[b] TGI = Tumor growth inhibition.
[c] BWL = Body weight loss.

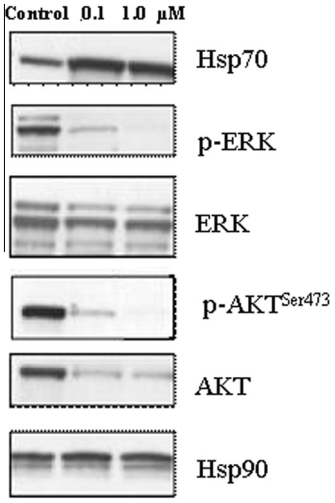


Figure 4. In vitro biomarkers modulation of compound **18** in A2780 cells at two concentrations.

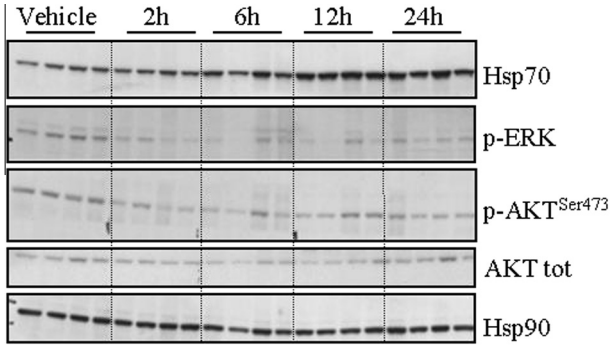


Figure 5. Ex vivo biomarkers modulation in lysates from A2780 tumors (3 tumors/group) isolated at different post-treatment times after single iv administration of 60 mg/kg of compound **18**.

4. Conclusion

The application of a fragment based approach and subsequent optimization led to the identification of compound **18** (NMS-E973), which showed nanomolar affinity to Hsp90 and high selectivity towards a panel of kinases, as well as against the biologically related ATPase Hsc70. In vivo data in a mouse model of human ovarian cancer demonstrated that the antitumor activity of compound **18** is related to the inhibition of Hsp90, with a favorable pharmacokinetic and safety profile. NMS-E973 was selected for further characterization in additional tumor models that have been presented in a paper submitted separately.[27]

5. Experimental

5.1. Chemistry

All solvents and reagents, unless otherwise stated, were commercially available, of the best grade and were used without further purification. All experiments dealing with moisture-sensitive compounds were carried out under dry nitrogen or argon atmosphere. Thin-layer chromatography was performed on Merck Silica Gel 60 F_{254} pre-coated plates. Column chromatography was conducted either under medium pressure on silica (Merck Silica Gel 40–63 μm) or performed by using a Biotage SP1 flash purification system with prepacked silica gel cartridges (Biotage or Varian). Components were visualized by UV light (λ: 254 nm) and by iodine vapor. ^{1}H NMR spectra were recorded at a constant temperature of 28 °C on a Varian INOVA 400 spectrometer (operating at 400.5 MHz for ^{1}H) and equipped with a 5 mm ^{1}H{^{13}C,^{15}N} z axis PFG indirect detection probe. Chemical shifts were referenced with respect to the residual solvents signals. Data are reported as follows: chemical shift (δ), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br s = broad signal, td = triplet of doublet, dd = doublet of doublets, ddd = doublet of doublets of doublets, m = multiplet), coupling constants (Hz), and number of protons. Electrospray (ESI) mass spectra were obtained on a Finnigan LCQ ion trap. HPLC–UV–MS analyses were carried out combining the ion trap MS instrument with HPLC system SSP4000 (Thermo Separation Products) equipped with an autosampler LC Pal (CTC Analytics) and UV6000LP diode array detector (UV detection 215–400 nm). Instrument control, data acquisition and processing were performed by using Xcalibur 1.2 software (Finnigan). HPLC chromatography was run at room temperature, and 1 mL/min flow rate, using a Waters X Terra RP 18 column (4.6 × 50 mm; 3.5 μm). Mobile phase A was ammonium acetate 5 mM buffer (pH 5.5 with acetic acid)/acetonitrile 90:10, and mobile phase B was ammonium acetate 5 mM buffer (pH 5.5 with acetic acid)/acetonitrile 10:90; the gradient was from 0% to 100% B in 7 min then hold 100% B for 2 min before requilibration. Mass data, given as m/z ratio. ESI(+) high resolution mass spectra (HRMS), were obtained on a Waters Q-Tof Ultima directly connected with a micro HPLC 1100 Agilent as previously described.[28]

5.1.1. 5-(3,4-Dichlorophenoxy)benzene-1,3-diol (1)

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.48 (br s, 2H), 7.57 (d, J = 8.8 Hz, 1H), 7.23 (d, J = 2.8 Hz, 1H), 6.96 (dd, J = 2.8, 8.8 Hz, 1H), 6.00 (t, J = 2.1 Hz, 1H), 5.83 (d, J = 2.1 Hz, 2H); LC–MS (ESI): m/z 270 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{12}H_8Cl_2O_3$+H^+ 270.9923, found 270.9928.

5.1.2. 1-[2,4-Dimethoxy-6-(4-nitrophenoxy)phenyl] ethanone (40a)

A stirred suspension of 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone (**39a**, 10.0 g, 51 mmol), potassium carbonate (10.48 g, 76 mmol) and 1-fluoro-4-nitrobenzene (5.4 mL, 51 mmol) in DMSO (50 mL) was heated at 110 °C for 2 h. The reaction mixture was diluted with EtOAc (100 mL) and thoroughly washed with brine (4 × 50 mL). The organic phase was dried over Na_2SO_4 and then evaporated to dryness. The crude was purified by Biotage SP1 flash chromatography (gradient elution from 3% to 20% of EtOAc in hexane) to afford **40a** (11.9 g, 63%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.23 (d, J = 9.3 Hz, 2H), 7.09 (m, 2H), 6.62 (d, J = 2.2 Hz, 1H), 6.36 (d, J = 2.1 Hz, 1H), 3.86 (s, 3H), 3.78 (s, 3H), 2.33 (s, 3H); LC–MS (ESI): m/z 318 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{16}H_{15}NO_6$+H^+ 318.0972, found 318.0973.

The following compounds **40b** and **40c** were prepared according to the method described above.

5.1.3. 1-[2-Methoxy-6-(4-nitrophenoxy)phenyl] ethanone (40b)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.24 (d, J = 9.3 Hz, 2H), 7.49 (t, J = 8.2 Hz, 1H), 7.10 (d, J = 9.3 Hz, 2H), 7.07 (d, J = 8.2 Hz, 1H), 6.76 (dd, J = 8.3, 0.6 Hz, 1H), 3.87 (s, 3H), 2.38 (s, 3H); LC–MS (ESI): m/z 288 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{15}H_{13}NO_5$+H^+ 288.0867, found 288.0871.

5.1.4. 1-[4-Methoxy-2-(4-nitrophenoxy)phenyl] ethanone (40c)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (d, J = 9.4 Hz, 2H), 7.92 (d, J = 8.8 Hz, 1H), 7.14 (d, J = 9.4 Hz, 2H), 7.01 (dd, J = 8.8, 2.5 Hz, 1H), 6.78 (d, J = 2.4 Hz, 1H), 3.82 (s, 3H), 2.42 (s, 3H); LC–MS (ESI): m/z

288 $[M+H]^+$; HRMS (ESI): *m/z* calcd for $C_{15}H_{13}NO_5+H^+$ 288.0867, found 288.0872.

5.1.5. 1-[2-(4-Chlorophenoxy)-4,6-dimethoxy-phenyl]-ethanone (41)

To a solution of **40a** (2.2 g, 6.94 mmol) in MeOH/EtOAc/DCM (1:1:1, 30 mL) 37% HCl (1.15 mL) and Pd/C (1.0 g) were added. The reaction mixture was shaken at room temperature in a hydrogen athmosphere (40 psi) for 8 h and then filtered through a pad of Celite that was washed with MeOH. The filtrate was concentrated, triturated with ether and filtered to afford 1-[2-(4-amino-phenoxy)-4,6-dimethoxy-phenyl]-ethanone hydrochloride (1.98 g, 88%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.21 (br s, 3H), 7.19 (d, J = 8.7 Hz, 2H), 7.00 (d, J = 8. 7 Hz, 2H), 6.49 (d, J = 2.1 Hz, 1H), 6.06 (d, J = 2.1 Hz, 1H), 3.83 (s, 3H), 3.73 (s, 3H), 2.35 (s, 3H); LC–MS (ESI): *m/z* 288 $[M+H]^+$. This intermediate (1.0 g, 3.09 mmol) was added to a mixture of copper(II) chloride (771 mg, 4.52 mmol) and *tert*-butylnitrite (0.372 mL, 3.13 mmol) in acetonitrile (40 mL) heated at 65 °C. The reaction mixture was stirred under reflux for 2 h, then cooled to room temperature, poured into water and extracted with EtOAc (3 × 50 mL). The organic phase was dried over Na_2SO_4 and then evaporated to dryness. The crude was purified by Biotage SP1 flash chromatography (gradient elution from 3% to 20% of EtOAc in hexane) to afford **41** (823 mg, 87%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.37 (m, 2H), 6.99 (m, 2H), 6.27 (d, J = 2.4 Hz, 1H), 6.12 (d, J = 2.4 Hz, 1H), 3.84 (s, 3H), 3.80 (s, 3H), 2.37 (s, 3H); LC–MS (ESI): *m/z* 307 $[M+H]^+$; HRMS (ESI): *m/z* calcd for $C_{16}H_{15}ClO_4+H^+$ 307.7506, found 307.7509.

5.1.6. Ethyl 5-[2,4-dimethoxy-6-(4-nitrophenoxy)-phenyl]-isoxazole-3-carboxylate (43a)

To a stirred solution of **40a** (3.48 g, 11 mmol) in THF (50 mL) at -50 °C was added dropwise 1 M lithium bis(trimethylsilyl)amide in THF (12 mL, 12 mmol). After 30 min, a solution of diethyl oxalate (1.62 mL, 12 mmol) in THF (15 mL) was slowly added and the reaction mixture was stirred for 2 h at room temperature. The solution was poured into water (50 mL) and treated with 1 M HCl solution (13 mL), then extracted with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4 and the solvent removed. The crude dissolved in EtOH (50 mL) was treated with hydroxylamine hydrochloride (0.74 g, 15 mmol) and refluxed for 2 h. The solution was concentrated to a small volume and the precipitate was collected to provide **43a** (3.5 g, 78%, 2 steps). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.22 (m, 2H), 7.12 (m, 2H), 6.90 (s, 1H), 6.74 (d, J = 2.2 Hz, 1H), 6.53 (d, J = 2.3 Hz, 1H), 4.33 (q, J = 7.2 Hz, 2H), 3.91 (s, 3H), 3.84 (s, 3H), 1.29 (t, J = 7.1 Hz, 3H); LC–MS (ESI): *m/z* 415 $[M+H]^+$; HRMS (ESI): *m/z* calcd for $C_{20}H_{18}N_2O_8+H^+$ 415.1136, found 415.1136.

The following compounds **42**, **43b** and **43c** were prepared according to the method described above from **41**, **40b** and **40c**.

5.1.7. Ethyl 5-[2-(4-chlorophenoxy)-4,6-dimethoxyphenyl]-isoxazole-3-carboxylate (42)

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.41 (m, 2H), 7.01 (m, 2H), 6.90 (s, 1H), 6.63 (d, J = 2.2 Hz, 1H), 6.24 (d, J = 2.2 Hz, 1H), 4.36 (q, J = 7.1 Hz, 2H), 3.88 (s, 3H), 3.80 (s, 3H), 1.31 (t, J = 7.1 Hz, 3H); LC–MS (ESI): *m/z* 404 $[M+H]^+$; HRMS (ESI): *m/z* calcd for $C_{20}H_{18}ClNO_6+H^+$ 404.0896, found 404.08965.

5.1.8. Ethyl 5-[2-methoxy-6-(4-nitrophenoxy) phenyl]-isoxazole-3-carboxylate (43b)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.22 (d, J = 9.4 Hz, 2H), 7.66 (t, J = 8.4 Hz, 1H), 7.20 (dd, J = 8.5, 0.6 Hz, 1H), 7.12 (d, J = 9.4 Hz, 2H), 7.02 (s, 1H), 6.91 (dd, J = 8.2, 0.8 Hz, 1H), 4.35 (q, J = 7.1 Hz, 2H), 3.91 (s, 3H), 1.29 (t, J = 7.1 Hz, 3H); LC–MS (ESI): *m/z* 385 $[M+H]^+$; HRMS (ESI): *m/z* calcd for $C_{19}H_{16}N_2O_7+H^+$ 385.1031, found 385.1035.

5.1.9. Ethyl 5-[4-methoxy-2-(4-nitrophenoxy) phenyl]-isoxazole-3-carboxylate (43c)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.24–8.28 (m, 2H), 8.05 (d, J = 8.8 Hz 1H), 7.25 (m, 2H), 7.11 (dd, J = 8.8, 2.5 Hz, 1H), 6.94 (s, 1H), 6.91 (d, J = 2.4 Hz, 1H), 4.35 (q, J = 7.1 Hz, 2H), 3.84 (s, 3H), 1.30 (t, J = 7.1 Hz, 3H); LC–MS (ESI): *m/z* 385 $[M+H]^+$; HRMS (ESI): *m/z* calcd for $C_{19}H_{16}N_2O_7+H^+$ 385.1031, found 385.1026.

5.1.10. 5-[2,4-Dimethoxy-6-(4-nitrophenoxy)phenyl] -*N*-ethyl-isoxazole-3-carboxamide (45a)

A solution of **43a** (828 mg, 2.0 mmol) in 2 M ethylamine in THF solution (20 mL) in a stopped flask was heated at 100 °C for 5 h. The solvent was removed and the residue was crystallized from a small volume of MeOH to provide **45a** (770 mg, 93%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.69 (t, J = 5.7 Hz, 1H), 8.22 (m, 2H), 7.12 (m, 2H), 6.81 (s, 1H), 6.75 (d, J = 2.3 Hz, 1H), 6.54 (d, J = 2.2 Hz, 1H), 3.92 (s, 3H), 3.84 (s, 3H), 3.23 (m, 2H), 1.08 (t, J = 7.2 Hz, 3H); LC–MS (ESI): *m/z* 414 $[M+H]^+$; HRMS (ESI): *m/z* calcd for $C_{20}H_{19}N_3O_7+H^+$ 414.1296, found 414.1295.

The following compounds **44**, **45b** and **45c** were prepared according to the method described above from **42**, **43b** and **43c**.

5.1.11. 5-[2-(4-Chlorophenoxy)-4,6-dimethoxy-phenyl]-*N*-ethyl-isoxazole-3-carboxamide (44)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.69 (t, J = 5.7 Hz, 1H), 7.39 (m, 2H), 6.99 (m, 2H), 6.78 (s, 1H), 6.62 (d, J = 2.2 Hz, 1H), 6.24 (d, J = 2.2 Hz, 1H), 3.87 (s, 3H), 3.78 (s, 3H), 3.24 (m, 2H), 1.09 (t, J = 7.2 Hz, 3H); LC–MS (ESI): *m/z* 403 $[M+H]^+$; HRMS (ESI): *m/z* calcd for $C_{20}H_{19}ClN_2O_5+H^+$ 403.1056, found 403.1061.

5.1.12. *N*-Ethyl-5-[2-methoxy-6-(4-nitrophenoxy) phenyl]-isoxazole-3-carboxamide (45b)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.72 (t, J = 5.73 Hz, 1H), 8.22 (m, 2H), 7.46 (t, J = 8.2 Hz, 1H), 7.20 (dd, J = 8.3, 0.9 Hz, 1H), 7.11 (m, 2H), 702 (s, 1H), 6.90 (dd, J = 8.3, 0.9 Hz, 1H), 3.91 (s, 3H), 3.24 (m, 2H), 1.09 (t, J = 7.2 Hz, 3H); LC–MS (ESI): *m/z* 384 $[M+H]^+$; HRMS (ESI): *m/z* calcd for $C_{19}H_{17}N_3O_6+H^+$ 384.1190, found 384.1193.

5.1.13. *N*-Ethyl-5-[4-methoxy-2-(4-nitrophenoxy) phenyl]-isoxazole-3-carboxamide (45c)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.74 (t, J = 5.7 Hz, 1H), 8.27 (d, J = 9.4 Hz, 2H), 8.02 (d, J = 8.8 Hz, 1H), 7.22 (d, J = 9.4 Hz, 2H), 7.10 (dd, J = 8.8, 2.5 Hz, 1H), 6.90 (d, J = 2.5 Hz, 1H), 6.85 (s, 1H), 3.83 (s, 3H), 3.23 (qd, J = 7.1, 6.0 Hz, 2H), 1.08 (t, J = 7.2 Hz, 3H); LC–MS (ESI): *m/z* 384 $[M+H]^+$; HRMS (ESI): *m/z* calcd for $C_{19}H_{17}N_3O_6+H^+$ 384.1190, found 384.1189.

5.1.14. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy) phenyl]-*N*-ethyl-isoxazole-3-carboxamide (3)

To a stirred solution of **45a** (670 mg, 1.62 mmol) in DCM (5 mL) was slowly added 1 M BBr_3 in DCM (4.86 mL, 4.86 mmol) at 0 °C. After stirring for 60 h at room temperature, 1 M BBr_3 in DCM (4.86 mL, 4.86 mmol) was slowly added. After stirring for 60 h at room temperature, the cloudy solution was concentrated and then diluted with EtOAc and thoroughly washed with water, then with 1 M sodium hydrogen carbonate solution and dried over Na_2SO_4. The solvent was removed and the residue was purified by Biotage SP1 flash chromatography (gradient elution from 0% to 10% of MeOH in DCM) to afford **3** (305 mg, 48%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.71 (s, 1H), 10.30 (s, 1H), 8.65 (t, 1H), 8.21 (d, J = 9.4 Hz, 2H), 7.10 (d, J = 9.4 Hz, 2H), 6.79 (s, 1H), 6.45 (d, J = 2.2 Hz, 1H), 6.10 (d, J = 2.2 Hz, 1H), 3.22 (qd, J = 7.2, 5.8 Hz, 2H), 1.07 (t, J = 7.2 Hz, 3H); LC–MS (ESI): *m/z* 386 $[M+H]^+$; HRMS (ESI): *m/z* calcd for $C_{18}H_{15}N_3O_7+H^+$ 386.0983, found 386.0982.

The following compounds **2**, **4** and **5** were prepared according to the method described above from **44**, **45b** and **45c**.

5.1.15. 5-[2-(4-Chlorophenoxy)-4,6-dihydroxy-phenyl]-*N*-ethyl-isoxazole-3-carboxamide (2)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.47 (s, 1H), 10.07 (s, 1H), 8.66 (t, J = 5.8 Hz, 1H), 7.41 (m, 2H), 7.01 (m, 2H), 6.77 (m, 1H), 6.32 (d, J = 2.3 Hz, 1H), 5.87 (d, J = 2.3 Hz, 1H), 3.27 (m, 2H), 1.10 (t, J = 7.1 Hz, 3H); LC–MS (ESI): m/z 375 [M+H]$^+$; HRMS (ESI): m/z calcd for $C_{18}H_{15}ClN_2O_5$+H$^+$ 375.0743, found 375.0748.

5.1.16. *N*-Ethyl-5-[2-hydroxy-6-(4-nitrophenoxy) phenyl]-isoxazole-3-carboxamide (4)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.88 (s, 1H), 8.72 (t, J = 5.7 Hz, 1H), 8.23 (m, 2H), 7.46 (t, J = 8.2 Hz, 1H), 7.10 (m, 2H), 7.01 (dd, J = 8.4, 0.9 Hz, 1H), 6.95 (s, 1H), 6.75 (dd, J = 8.2, 0.8 Hz, 1H), 3.24 (m, 2H), 1.09 (t, J = 7.2 Hz, 3H); LC–MS (ESI): m/z 370 [M+H]$^+$; HRMS (ESI): m/z calcd for $C_{18}H_{15}N_3O_6$+H$^+$ 370.1034, found 370.1036

5.1.17. *N*-Ethyl-5-[4-hydroxy-2-(4-nitrophenoxy) phenyl]-isoxazole-3-carboxamide (5)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.55 (s, 1H), 8.72 (t, J = 5.5, 1H), 8.28 (d, J = 9.4 Hz, 2H), 7.90 (d, J = 8.7 Hz, 1H), 7.24 (d, J = 9.3 Hz, 2H), 6.89 (dd, J = 8.7, 2.4 Hz, 1H), 6.79 (s, 1H), 6.59 (d, J = 2.3 Hz, 1H), 3.23 (qd, J = 7.2, 5.7 Hz, 2H), 1.08 (t, 3H); LC–MS (ESI): m/z 370 [M+H]$^+$; HRMS (ESI): m/z calcd for $C_{18}H_{15}N_3O_6$+H$^+$ 370.1034, found 370.1037.

5.1.18. 5-[2-(4-Chlorophenoxy)-4,6-dimethoxy-phenyl]-isoxazole (46)

To a stirred solution of **41** (306 mg, 1.0 mmol) in THF (5 mL) at −50 °C was added dropwise 1 M lithium bis(trimethylsilyl)amide in THF (1.1 mL, 1.1 mmol). After 30 min, a solution of ethyl formate (0.096 mL, 1.2 mmol) was slowly added and the reaction mixture was stirred for 2 h at room temperature. The solution was poured into water (10 mL) and treated with 1 M HCl solution (1.2 mL), extracted with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4 and the solvent removed. The crude dissolved in ethanol (5 mL) was treated with hydroxylamine hydrochloride (90.0 mg, 1.3 mmol) and refluxed for 2 h. The solution was concentrated to a small volume and the precipitate was collected to provide **46** (268 mg, 81%, 2 steps). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.51 (d, J = 1.9 Hz, 1H), 7.38 (m, 2H), 6.95 (m, 2H), 6.61 (d, J = 2.3 Hz, 1H), 6.50 (d, J = 1.8 Hz, 1H), 6.26 (d, J = 2.3 Hz, 1H), 3.86 (s, 3H), 3.79 (s, 3H); LC–MS (ESI): m/z 332 [M+H]$^+$; HRMS (ESI): m/z calcd for $C_{17}H_{14}ClNO_4$+H$^+$ 332.0684, found 332.0688.

5.1.19. 5-(4-Chlorophenoxy)-4-(isoxazol-5-yl) benzene-1,3-diol (6)

To a stirred solution of **46** (87 mg, 0.26 mmol) in DCM (2 mL) was slowly added 1 M BBr_3 in DCM (0.78 mL, 0.78 mmol) at 0 °C. After stirring for 120 h at room temperature, the cloudy solution was diluted with DCM and thoroughly washed with water, then with 1 M sodium hydrogen carbonate solution and dried over Na_2SO_4. The solvent was removed and the residue was purified by Biotage SP1 flash chromatography (gradient elution from 0% to 10% of MeOH in DCM) to afford **6** (36 mg, 45%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.29 (s, 1H), 9.97 (s, 1H), 8.45 (d, J = 1.8 Hz, 1H), 7.38 (d, J = 9.0 Hz, 2H), 6.97 (d, J = 9.0 Hz, 2H), 6.49 (d, J = 1.8 Hz, 1H), 6.30 (d, J = 2.3 Hz, 1H), 5.86 (d, J = 2.2 Hz, 1H); LC–MS (ESI): m/z 304 [M+H]$^+$; HRMS (ESI): m/z calcd for $C_{15}H_{10}ClNO_4$+H$^+$ 304.0371, found 304.0374.

5.1.20. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy)phenyl] -isoxazole-3-carboxylic acid (47)

To a stirred solution of **43a** (1.9 g, 4.6 mmol) in DCM (20 mL) was slowly added 1 M BBr_3 in DCM (23 mL, 23 mmol) at 0 °C. After stirring for 60 h at room temperature, 1 M BBr_3 in DCM (23 mL, 23 mmol) was slowly added. After stirring for other 60 h at room temperature, the cloudy solution was diluted with DCM and washed with 1 M sodium hydrogen carbonate solution. The aqueous solution was acidified with citric acid to pH 5 and thoroughly extracted with EtOAc, and dried over Na_2SO_4. The solvent was removed and the residue was purified twice by Biotage SP1 flash chromatography (gradient elution from 2% to 20% of MeOH in DCM) to afford **47** (480 mg, 29%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.72 (s, 1H), 10.31 (s, 1H), 8.22 (d, J = 9.3 Hz, 2H), 7.11 (d, J = 9.3 Hz, 2H), 6.81 (s, 1H), 6.45 (d, J = 2.2 Hz, 1H), 6.09 (d, J = 2.3 Hz, 1H); LC–MS (ESI): m/z 359 [M+H]$^+$; HRMS (ESI): m/z calcd for $C_{16}H_{10}N_2O_8$+H$^+$ 359.0510, found 359.0514.

5.1.21. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy) phenyl]-*N*-(2-fluoroethyl)-isoxazole-3-carboxamide (8)

To a stirred solution of **47** (107 mg, 0.3 mmol) in dimethylacetamide (3 mL) was added *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU) (115 mg, 0.36 mmol) After stirring for 15 min at room temperature, *N*,*N*-diisopropylethylamine (0.104 mL, 0.6 mmol) and 2-fluoroethylamine hydrochloride (45 mg, 0.45 mmol) were added. After stirring overnight, the reaction mixture was poured into a saturated solution of sodium hydrogen carbonate and thoroughly extracted with EtOAc, washed with brine, dried over Na_2SO_4 and evaporated to dryness. The residue was purified by Biotage SP1 flash chromatography (gradient elution from 2% to 10% of MeOH in DCM) to afford **8** (86 mg, 71%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.73 (s, 1H), 10.31 (s, 1H), 8.83 (t, 1H), 8.22 (d, J = 9.3 Hz, 2H), 7.11 (d, J = 9.3 Hz, 2H), 6.82 (s, 1H), 6.46 (d, J = 2.2 Hz, 1H), 6.10 (d, J = 2.2 Hz, 1H), 4.49 (dt, $J_{(H,F)}$ = 47.4 Hz, J = 5.1 Hz, 2H), 3.54 (q, J = 5.3 Hz, 1H), 3.48 (q, J = 5.3 Hz, 1H); LC–MS (ESI): m/z 404 [M+H]$^+$; HRMS (ESI): m/z calcd for $C_{18}H_{14}FN_3O_7$+H$^+$ 404.0889, found 404.0885.

The following compounds **7**, **9**–**16**, **18**–**20**, **29** were prepared according to the method described above using the suitable amine.

5.1.22. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy) phenyl]-*N*,*N*-dimethyl-isoxazole-3-carboxamide (7)

^{1}H NMR^{1}H NMR (400 MHz, DMSO-d_6) δ 10.67 (br s, 1H), 10.30 (br s, 1H), 8.20 (d, J = 9.4 Hz, 2H), 7.10 (d, J = 9.4 Hz, 2H), 6.68 (s, 1H), 6.44 (d, J = 2.2 Hz, 1H), 6.10 (d, J = 2.3 Hz, 1H), 2.96 (s, 3H), 2.95 (s, 3H); LC–MS (ESI): m/z 386 [M+H]$^+$; HRMS (ESI): m/z calcd for $C_{18}H_{15}N_3O_7$+H$^+$ 386.0983, found 386.0991.

5.1.23. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy) phenyl]-*N*-(2-methoxyethyl)-isoxazole-3-carboxamide (9)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.72 (s, 1H), 10.30 (s, 1H), 8.60 (t, J = 5.4 Hz, 1H), 8.21 (m, 2H), 7.10 (m, 2H), 6.81 (s, 1H), 6.45 (d, J = 2.2 Hz, 1H), 6.10 (d, J = 2.2 Hz, 1H), 3.40 (m, 4H), 3.23 (s, 3H); LC–MS (ESI): m/z 416 [M+H]$^+$; HRMS (ESI): m/z calcd for $C_{19}H_{17}N_3O_8$+H$^+$ 416.1089, found 416.1093.

5.1.24. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy) phenyl]-*N*-(2-hydroxyethyl)-isoxazole-3-carboxamide (10)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.72 (br s, 1H), 10.31 (br s, 1H), 8.50 (t, J = 5.7 Hz, 1H), 8.21 (m, 2H), 7.10 (m, 2H), 6.81 (s, 1H), 6.45 (d, J = 2.2 Hz, 1H), 6.10 (d, J = 2.2 Hz, 1H), 4.69 (t, J = 5.6 Hz, 1H), 3.46 (q, J = 6.1 Hz, 2H), 3.29 (m, 2H); LC–MS (ESI): m/z 402 [M+H]$^+$; HRMS (ESI): m/z calcd for $C_{18}H_{15}N_3O_8$+H$^+$ 402.0932, found 402.0931.

5.1.25. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy) phenyl]-*N*-[3-(dimethylamino)propyl]-isoxazole-3-carboxamide (11)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.75 (s, 1H), 10.34 (s, 1H), 9.19 (br s, 1H), 8.80 (t, J = 5.8 Hz, 1H), 8.22 (m, 2H), 7.10 (m, 2H), 6.81 (s, 1H), 6.46 (d, J = 2.2 Hz, 1H), 6.11 (d, J = 2.3 Hz, 1H), 3.28 (m, 2H),

3.03 (m, 2H), 2.75 (d, J = 4.9 Hz, 6H), 1.81 (m, 2H); LC–MS (ESI): m/z 443 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{21}H_{22}N_4O_7+H^+$ 443.1562, found 443.1566.

5.1.26. *N*-Cyclohexyl-5-[2,4-dihydroxy-6-(4-nitrophenoxy)phenyl]-isoxazole-3-carboxamide (12)

^1H NMR (400 MHz, DMSO-d_6) δ 10.71 (s, 1H), 10.30 (s, 1H), 8.43 (d, J = 8.3 Hz, 1H), 8.22 (m, 2H), 7.10 (m, 2H), 6.79 (s, 1H), 6.45 (d, J = 2.2 Hz, 1H), 6.10 (d, J = 2.2 Hz, 1H), 3.61–3.75 (m, 1H), 1.66–1.76 (m, 4H), 1.53–1.60 (m, 1H), 1.21–1.33 (m, 4H), 1.05–1.12 (m, 1H); LC–MS (ESI): m/z 440 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{22}H_{21}N_3O_7+H^+$ 440.1453, found 440.1456.

5.1.27. *N*-Benzyl-5-[2,4-dihydroxy-6-(4-nitrophenoxy)phenyl]-isoxazole-3-carboxamide (13)

^1H NMR (400 MHz, DMSO-d_6) δ 10.72 (s, 1H), 10.31 (s, 1H), 9.23 (t, J = 6.2 Hz, 1H), 8.21 (d, J = 9.2 Hz, 2H), 7.11 (d, J = 9.2 Hz, 2H), 6.83 (s, 1H), 6.45 (d, J = 2.2 Hz, 1H), 6.10 (d, J = 2.2 Hz, 1H), 4.40 (d, J = 6.2 Hz, 2H); LC–MS (ESI): m/z 448 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{23}H_{17}N_3O_7+H^+$ 448.1140, found 448.1145.

5.1.28. *N*-(Cyclohexylmethyl)-5-[2,4-dihydroxy-6-(4-nitrophenoxy)phenyl]-isoxazole-3-carboxamide (14)

^1H NMR (400 MHz, DMSO-d_6) δ 10.71 (s, 1H), 10.30 (s, 1H), 8.60 (t, J = 6.1 Hz, 1H), 8.21 (d, J = 9.3 Hz, 2H), 7.10 (d, J = 9.3 Hz, 2H), 6.79 (s, 1H), 6.45 (d, J = 2.3 Hz, 1H), 6.10 (d, J = 2.2 Hz, 1H), 3.04 (t, J = 6.5 Hz, 2H), 1.64 (m, 5H), 1.50 (m, 1H), 1.14 (m, 4H), 0.87 (m, 1H); LC–MS (ESI): m/z 454 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{23}H_{23}N_3O_7+H^+$ 454.1609, found 454.1608.

5.1.29. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy) phenyl]-*N*-(piperidin-4-ylmethyl)-isoxazole-3-carboxamide trifluoroacetate (15)

Compound **15** was obtained from *tert*-butyl 4-{[({5-[2,4-dihydroxy-6-(4-nitrophenoxy) phenyl]-isoxazol-3-yl}carbonyl)amino]methyl} piperidine-1-carboxylate (prepared according to the method described above) after treatment with 50% CF_3COOH in DCM. ^1H NMR (400 MHz, DMSO-d_6) δ 10.74 (s, 1H), 10.33 (s, 1H), 8.77 (t, J = 6.1 Hz, 1H), 8.42 (d, J = 9.6 Hz, 1H), 8.22 (d, J = 9.3 Hz, 2H), 8.07 (d, J = 9.9 Hz, 1H), 7.10 (d, J = 9.3 Hz, 2H), 6.80 (s, 1H), 6.46 (d, J = 2.3 Hz, 1H), 6.11 (d, J = 2.2 Hz, 1H), 3.24 (m, 2H), 3.13 (t, J = 6.3 Hz, 2H), 2.82 (m, 2H), 1.82 (m, 1H), 1.76 (m, 2H), 1.28 (m, 2H); LC–MS (ESI): m/z 455 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{22}H_{22}N_4O_7+H^+$ 455.1562, found 455.1564.

5.1.30. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy) phenyl]-*N*-[(1-methylpiperidin-4-yl)methyl]-isoxazole-3-carboxamide (16)

^1H NMR (400 MHz, DMSO-d_6) δ 10.75 (s, 1H), 10.34 (s, 1H), 8.77 (t, J = 6.0 Hz, 1H), 8.22 (m, 2H), 7.10 (m, 2H), 6.80 (s, 1H), 6.46 (d, J = 2.2 Hz, 1H), 6.11 (d, J = 2.3 Hz, 1H), 3.26–3.46 (m, 2H), 3.12 (t, J = 6.3 Hz, 2H), 2.88 (m, 2H), 2.25 (s, 3H), 1.79 (m, 2H), 1.74 (m, 1H), 1.29 (m, 2H); LC–MS (ESI): m/z 469 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{23}H_{24}N_4O_7+H^+$ 469.1718, found 469.1722.

5.1.31. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy) phenyl]-*N*-(1-methylpiperidin-4-yl)-isoxazole-3-carboxamide (18)

^1H NMR (400 MHz, DMSO-d_6) δ 10.71 (br s, 1H), 10.31 (br s, 1H), 8.54 (d, J = 7.9 Hz, 1H), 8.22 (m, 2H), 7.10 (m, 2H), 6.80 (s, 1H), 6.46 (d, J = 2.2 Hz, 1H), 6.10 (d, J = 2.2 Hz, 1H), 3.70 (m, 1H), 2.80 (m, 2H), 2.21 (s, 3H), 2.01 (m, 2H), 1.72 (m, 2H), 1.61 (m, 2H); LC–MS (ESI): m/z 455 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{22}H_{22}N_4O_7+H^+$ 455.1562, found 455.1563.

5.1.32. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy) phenyl]-*N*-(1,2,2,6,6-pentamethylpiperidin-4-yl)-isoxazole-3-carboxamide (19)

^1H NMR (400 MHz, DMSO-d_6) δ 10.75 (br s, 1H), 10.33 (br s, 1H), 8.17–8.27 (m, 2H), 7.04–7.15 (m, 2H), 6.80 (s, 1H), 6.47 (d, J = 2.2 Hz, 1H), 6.11 (d, J = 2.2 Hz, 1H), 4.09–4.32 (m, 1H), 2.73 (br s, 2H), 1.53–2.27 (m, 8H), 0.96–1.50 (m, 12H); LC–MS (ESI): m/z 511 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{26}H_{30}N_4O_7+H^+$ 511.2188, found 511.2176.

5.1.33. *N*-(1-Acetylpiperidin-4-yl)-5-[2,4-dihydroxy-6-(4-nitrophenoxy)phenyl]-isoxazole-3-carboxamide (20)

^1H NMR (400 MHz, DMSO-d_6) δ 10.72 (s, 1H), 10.31 (s, 1H), 8.60 (d, J = 7.9 Hz, 1H), 8.22 (d, J = 9.3 Hz, 2H), 7.10 (d, J = 9.3 Hz, 2H), 6.81 (s, 1H), 6.46 (d, J = 2.2 Hz, 1H), 6.10 (d, J = 2.3 Hz, 1H), 4.31 (m, 1H), 3.96 (m, 1H), 3.79 (m, 1H), 3.08 (m, 1H), 2.62 (td, J = 12.7, 2.9 Hz, 1H), 1.98 (s, 3H), 1.73 (m, 2H), 1.46 (m, 1H), 1.37 (m, 1H); LC–MS (ESI): m/z 483 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{23}H_{22}N_4O_8+H^+$ 483.1511, found 483.1517.

5.1.34. *N*-(1-Benzylpiperidin-4-yl)-5-[2,4-dihydroxy-6-(4-nitrophenoxy)phenyl]-isoxazole-3-carboxamide (29)

^1H NMR (400 MHz, DMSO-d_6) δ 10.74 (br s, 1H), 10.33 (s, 1H), 8.21 (d, J = 9.3 Hz, 2H), 7.18–7.63 (br s, 5H),7.09 (d, J = 9.3 Hz, 2H), 6.80 (s, 1H), 6.47 (d, J = 2.2 Hz, 1H), 6.10 (d, J = 2.2 Hz, 1H), 3.70 (m, 1H), 2.80 (m, 2H), 2.21 (s, 3H), 2.01 (m, 2H), 1.72 (m, 2H), 1.61 (m, 2H); LC–MS (ESI): m/z 531 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{28}H_{26}N_4O_7+H^+$ 531.1875, found 531.1881.

5.1.35. 1-[2-Hydroxy-4,6-bis(methoxymethoxy) phenyl]ethanone (49)

To a stirred solution of dimethoxymethane (66 mL, 745 mmol) and zinc bromide (447 mg, 1.12 mmol) in DCM (580 mL) was added dropwise acetyl chloride (53 mL, 745 mmol) during 30 min maintaining the temperature below 30 °C. After stirring 3 h at room temperature, the solution was diluted with DCM (1.2 L), then cooled at 5 °C before the portion wise addition of 1-(2,4,6-trihydroxyphenyl)ethanone (**48**, 50.0 g, 298 mmol) followed by the dropwise addition of *N*,*N*-diisopropylethylamine (208 mL, 1.19 mol). After 1 h the ice bath was removed and the temperature was allowed to rise to room temperature. The resulting cloudy solution was stirred overnight, and then was washed with NH_4Cl saturated solution, followed by washing with 10% citric acid solution. After drying over Na_2SO_4, the solvent was removed to give **49** (78.0 g, 100%). ^1H NMR (400 MHz, DMSO-d_6) δ 13.31 (s, 1H), 6.24 (d, J = 2.3 Hz, 1H), 6.19 (d, J = 2.3 Hz, 1H), 6.19 (d, J = 2.3 Hz, 1H), 5.30 (s, 2H), 5.22 (s, 2H), 3.44 (s, 3H), 3.38 (s, 3H), 2.60 (s, 3H); LC–MS (ESI): m/z 257 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{12}H_{16}O_6+H^+$ 257.1020, found 257.1019.

5.1.36. 1-[2,4-Bis(methoxymethoxy)-6-(4-nitrophenoxy)phenyl]ethanone (50)

To a stirred solution of **49** (78.0 g, 298 mmol) in DMSO (500 mL) 4-nitro-1-fluorobenzene (46.3 g, 328 mmol) was added, followed by water (40 mL) and K_2CO_3 (45.3 g, 328 mmol). After stirring for 15 min at room temperature, the resulting suspension was heated for 6 h at 55 °C. After cooling, the dark solution was diluted with EtOAc (2.0 L) and thoroughly washed with 10% citric acid solution, then with brine and dried over Na_2SO_4. The solvent was removed and the residue was purified by flash chromatography (eluent: cyclohexane/EtOAc 3:1) to provide **50** (56.17 g, 50%). ^1H NMR (400 MHz, DMSO-d_6) δ 8.17–8.32 (m, 2H), 6.98–7.23 (m, 2H), 6.76 (d, J = 2.1 Hz, 1H), 6.47 (d, J = 2.1 Hz, 1H), 5.28 (s, 2H), 5.19 (s, 2H), 3.41 (s, 3H), 3.37 (s, 3H), 2.38 (s, 3H); LC–MS (ESI): m/z 378 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{18}H_{19}NO_8+H^+$ 378.1184, found 378.1187.

5.1.37. Ethyl 5-[2,4-bis(methoxymethoxy)-6-(4-nitrophenoxy)phenyl]-isoxazole-3-carboxylate (51)

To a stirred solution of sodium *tert*-butoxide (31.5 g, 328 mmol) in THF (250 mL) at −10 °C diethyl oxalate (60.5 mL, 447 mmol) in

250 mL of precooled THF was added dropwise. After 30 min a solution of **50** (56.0 g, 149 mmol) in THF (350 mL) was added dropwise. The reaction mixture was stirred for 1 h at −10 °C and then for further 2 h at room temperature. The solution was poured into a 10% citric acid solution (600 mL) and thoroughly extracted with EtOAc. After washing with brine and drying over Na_2SO_4, the solvent was removed to provide a yellowish residue that was taken up in light petrol ether to remove the excess of diethyl oxalate to afford the quite pure ethyl (2*E*)-4-[2,4-bis(methoxymethoxy)-6-(4-nitrophenoxy)phenyl]-2-hydroxy-4-oxobut-2-enoate, which was used directly in the next synthetic step. To a stirred solution of the crude (71.0 g, 149 mmol) in THF (250 mL) and 95° ethanol (500 mL) sodium acetate (24.4 g, 298 mmol) and hydroxylamine hydrochloride (22.8 g, 328 mmol) were added at room temperature. After 2 h, 6 M HCl solution was added dropwise to reach pH 2. After stirring for 24 h, saturated solution of Na_2HPO_4 was added to reach pH 6 and the suspension taken up in EtOAc was thoroughly washed with brine and dried over Na_2SO_4. The solvent was removed and the residue was crystallized from a mixture of MTBE (240 mL) and hexane (180 mL), to provide **51** (35.3 g, 50%, 2 steps). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.18–8.35 (m, 2H), 7.06–7.24 (m, 2H), 6.96 (s, 1H), 6.89 (d, J = 2.2 Hz, 1H), 6.62 (d, J = 2.2 Hz, 1H), 5.33 (s, 2H), 5.26 (s, 2H), 4.34 (q, J = 7.1 Hz, 2H), 3.39 (s, 3H), 3.38 (s, 3H), 1.29 (t, J = 7.1 Hz, 3H); LC–MS (ESI): m/z 475 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{22}H_{22}N_2O_{10}$+H^+ 475.1347, found 475.1346.

5.1.38. *tert*-Butyl 4-[({5-[2,4-bis(methoxymethoxy)-6-(4-nitrophenoxy)phenyl]-isoxazol-3-yl}carbonyl)amino]piperidine-1-carboxylate (52)

51 (5.0 g, 10.5 mmol) and *tert*-butyl 4-aminopiperidine-1-carboxylate (8.0 g, 40 mmol) were dissolved in DCM (30 mL). The solvent was removed and the reaction mixture was stirred at 80 °C for 24 h. After cooling to room temperature, the residue was purified by Biotage SP1 flash chromatography (hexane/EtOAc 80:20) to afford **52** (6.1 g, 97%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.65 (d, J = 8.2 Hz, 1H), 8.22 (d, J = 9.3 Hz, 2H), 7.13 (d, J = 9.3 Hz, 2H), 6.88 (d, J = 2.2 Hz, 1H), 6.86 (s, 1H), 6.62 (d, J = 2.3 Hz, 1H), 5.33 (s, 2H), 5.25 (s, 2H), 3.82–3.98 (m, 3H), 3.39 (s, 3H), 3.38 (s, 3H), 2.79 (br s, 2H), 1.71 (m, 2H), 1.39 (s, 9H), 1.41 (qd, J = 12.2, 4.4 Hz, 2H); LC–MS (ESI): m/z 629 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{30}H_{36}N_4O_{11}$+H^+ 629.2454, found 629.2451.

5.1.39. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy) phenyl]-*N*-(piperidin-4-yl)-isoxazole-3-carboxamide hydrochloride (17)

To a solution of **52** (2.0 g, 3.2 mmol) in ethanol (8 mL) was added 4 M HCl in dioxane (8 mL) at room temperature. The solution was set aside for 5 h then the solvent was evaporated off and the residue taken up in diethyl ether to provide **17** (1.41 g, 93%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.77 (s, 1H), 10.35 (s, 1H), 8.82 (d, J = 7.8 Hz, 1H), 8.60 (br s, 1H), 8.34 (br s, 1H), 8.22 (m, 2H), 7.19 (m, 2H), 6.83 (s, 1H), 6.49 (d, J = 2.2 Hz, 1H), 6.11 (d, J = 2.2 Hz, 1H), 4.05 (m, 1H), 3.25 (m, 2H), 2.98 (m, 2H), 1.91 (m, 2H), 1.72 (m, 2H); LC–MS (ESI): m/z 441 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{21}H_{20}N_4O_7$+H^+ 441.1405, found 441.1401.

5.1.40. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy) phenyl]-*N*-[1-(4,4,4-trifluorobutyl)piperidin-4-yl]-isoxazole-3-carboxamide (21)

A solution of **17** (100 mg, 0.23 mmol), *N*,*N*-diisopropylethylamine (0.157 mL, 0.92 mmol) and 1-bromo-4,4,4-trifluorobutane (0.036 mL, 0.27 mmol) in DMF (3 mL) was stirred at room temperature overnight. The solution was taken up in EtOAc and thoroughly washed with brine, then dried over Na_2SO_4. After removal of the solvent, the residue was purified by flash chromatography (eluent: DCM/MeOH/acetone 90:5:5) to provide **21** (56 mg, 44% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.72 (br s, 1H), 10.30 (br s, 1H), 8.51 (d, J = 7.9 Hz, 1H), 8.22 (d, J = 9.3 Hz, 2H), 7.10 (d, J = 9.3 Hz, 2H), 6.80 (s, 1H), 6.45 (d, J = 2.2 Hz, 1H), 6.10 (d, J = 2.2 Hz, 1H), 3.69 (m, 1H), 2.80 (m, 2H), 2.31 (t, J = 7.0 Hz, 2H), 2.15–2.28 (m, 2H), 1.94 (td, J = 11.7, 1.8 Hz, 2H), 1.70 (m, 2H), 1.61 (m, 2H), 1.55 (qd, J = 11.8, 3.2 Hz, 2H); LC–MS (ESI): m/z 551 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{25}H_{25}F_3N_4O_7$+H^+ 551.1748, found 551.1745.

5.1.41. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy) phenyl]-*N*-[1-(propan-2-yl)piperidin-4-yl]-isoxazole-3-carboxamide (22)

To a stirred solution of **17** (100 mg, 0.23 mmol) and acetone (0.033 mL, 0.46 mmol) in DMF (3 mL) was added portionwise tetramethylammonium triacetoxyborohydride (121 mg, 0.46 mmol). After stirring at room temperature overnight, the solution was taken up in EtOAc and thoroughly washed with brine. After drying over Na_2SO_4, the solvent was removed and the residue was purified by flash chromatography (gradient elution from 10% to 20% of MeOH in DCM) to provide 22 (81 mg, 73%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.77 (br s, 1H), 10.35 (br s, 1H), 8.63 (br s, 1H), 8.22 (m, 2H), 7.10 (m, 2H), 6.81 (s, 1H), 6.49 (d, J = 2.2 Hz, 1H), 6.11 (d, J = 2.2 Hz, 1H), 3.79 (m, 1H), 2.94 (br s, 3H), 1.56–1.87 (m, 6H), 1.04 (br s, 3H); LC–MS (ESI): m/z 483 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{24}H_{26}N_4O_7$+H^+ 483.1875, found 483.1872.

The following compounds **23**–**28** were prepared according to the method described above using the suitable aldehyde or ketone.

5.1.42. *N*-(1-Cyclohexylpiperidin-4-yl)-5-[2,4-dihydroxy-6-(4-nitrophenoxy)phenyl]-isoxazole-3-carboxamide (23)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.72 (br s, 1H), 10.30 (br s, 1H), 8.52 (br s, 1H), 8.22 (m, 2H), 7.10 (m, 2H), 6.80 (s, 1H), 6.46 (d, J = 2.2 Hz, 1H), 6.10 (d, J = 2.2 Hz, 1H), 3.69 (br s, 1H), 2.85 (br s, 2H), 2.27 (br s, 3H), 1.73 (m, 5H), 1.57 (m, 3H), 1.20 (m, 2H), 1.07 (m, 1H); LC–MS (ESI): m/z 523 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{27}H_{30}N_4O_7$+H^+ 523.2188, found 523.2190.

5.1.43. *N*-(1-Cyclopentylpiperidin-4-yl)-5-[2,4-dihydroxy-6-(4-nitrophenoxy)phenyl]-isoxazole-3-carboxamide (24)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.70 (br s, 1H), 10.31 (br s, 1H), 8.53 (d, J = 8.7 Hz, 1H), 8.22 (m, 2H), 7.10 (m, 2H), 6.80 (s, 1H), 6.46 (d, J = 2.2 Hz, 1H), 6.10 (d, J = 2.2 Hz, 1H), 3.71 (m, 1H), 2.94 (br s, 2H), 1.97 (br s, 2H), 1.17–1.82 (m, 13H); LC–MS (ESI): m/z 509 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{26}H_{28}N_4O_7$+H^+ 509.2031, found 509.2028.

5.1.44. *N*-(1-Cycloheptylpiperidin-4-yl)-5-[2,4-dihydroxy-6-(4-nitrophenoxy)phenyl]-isoxazole-3-carboxamide (25)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.73 (br s, 1H), 10.32 (br s, 1H), 8.54 (br s, 1H), 8.22 (m, 2H), 7.10 (m, 2H), 6.80 (s, 1H), 6.46 (d, J = 2.3 Hz, 1H), 6.10 (d, J = 2.3 Hz, 1H), 3.71 (br s, 1H), 2.77 (br s, 2H), 2.21 (br s, 1H), 1.24–1.88 (m, 16H); LC–MS (ESI): m/z 537 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{28}H_{32}N_4O_7$+H^+ 537.2344, found 537.2347.

5.1.45. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy)phenyl]-*N*-(1'-methyl-1,4'-bipiperidin-4-yl)-isoxazole-3-carboxamide (26)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.45 (d, J = 8.1 Hz, 1H), 8.21 (d, J = 9.3 Hz, 2H), 7.08 (d, J = 9.3 Hz, 2H), 6.86 (s, 1H), 6.37 (br s, 1H), 5.98 (br s, 1H), 3.66 (m, 1H), 2.82 (m, 2H), 2.76 (m, 2H), 2.12 (m, 2H), 2.11 (s, 3H), 1.80 (td, J = 11.9, 1.9 Hz, 2H), 1.59–1.73 (m, 4H), 1.50 (qd, J = 11.9, 3.9 Hz, 2H), 1.39 (qd, J = 12.1, 3.3 Hz, 2H); LC–MS (ESI): m/z 538 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{27}H_{31}N_5O_7$+H^+ 538.2297, found 538.2295.

5.1.46. 5-[2,4-Dihydroxy-6-(4-nitrophenoxy) phenyl]-*N*-[1-(1,4-dioxaspiro[4.5]dec-8-yl)piperidin-4-yl]-isoxazole-3-carboxamide (27)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.71 (br s, 1H), 10.30 (br s, 1H), 8.52 (br s, 1H), 8.22 (m, 2H), 7.10 (m, 2H), 6.80 (s, 1H), 6.46 (d,

J = 2.2 Hz, 1H), 6.10 (d, J = 2.2 Hz, 1H), 3.83 (s, 4H), 3.67 (br s, 1H), 2.80 (br s, 2H), 2.18 (br s, 2H), 1.41–1.75(m, 11H); LC–MS (ESI): m/z 581 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{29}H_{32}N_4O_9+H^+$ 581.2242, found 581.2238.

5.1.47. *N*-[1-(Cyclohexylmethyl)piperidin-4-yl]-5-[2,4-dihydroxy-6-(4-nitrophenoxy)phenyl]-isoxazole-3-carboxamide (28)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.75 (br s, 1H), 10.34 (s, 1H), 8.22 (d, J = 9.3 Hz, 1H), 7.10 (d, J = 9.3 Hz, 2H), 6.82 (s, 1H), 6.48 (d, J = 2.1 Hz, 1H), 6.11 (d, J = 2.1 Hz, 1H), 2.71–4.24 (m, 7H), 0.73–2.06 (m, 15H); LC–MS (ESI): m/z 537 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{28}H_{32}N_4O_7+H^++H^+$ 537.2344, found 537.2344.

5.1.48. 5-[2,4-Bis(methoxymethoxy)-6-(4-nitrophenoxy)phenyl]-*N*-(1-methylpiperidin-4-yl)-isoxazole-3-carboxamide (53)

51 (5.0 g, 10.5 mmol) and 1-methylpiperidin-4-amine (3.6 g, 31.5 mmol) were dissolved in DCM (30 mL). The solvent was removed and the reaction mixture was stirred at 80 °C for 9 h. After cooling to room temperature, the residue was purified by Biotage SP1 flash chromatography ((gradient elution from 0% to 10% of MeOH in DCM) to afford **53** (4.6 g, 80%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.66 (d, J = 7.8 Hz, 1H), 8.22 (m, 2H), 7.10 (m, 2H), 6.89 (d, J = 2.2 Hz, 1H), 6.85 (s, 1H), 6.63 (d, J = 2.2 Hz, 1H), 5.33 (s, 2H), 5.25 (s, 2H), 3.77 (br s, 1H), 3.39 (s, 3H), 3.38 (s, 3H), 2.90 (br s, 2H), 2.13–2.38 (m, 7H), 1.74 (m, 2H), 1.66 (m, 2H); LC–MS (ESI): m/z 543 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{26}H_{30}N_4O_9+H^+$ 543.2086, found 543.2083.

5.1.49. 5-[2-(4-Aminophenoxy)-4,6-bis(methoxymethoxy)phenyl]-*N*-(1-methylpiperidin-4-yl)-isoxazole-3-carboxamide (54)

To a stirred solution of **53** (2.41 g, 4.44 mmol) in a mixture of dioxane/water (5:1, 20 mL) were added portion wise zinc powder (575 mg, 8.88 mmol) and ammonium chloride (3.06 g, 44.4 mmol). After stirring at 100 °C for 3 h, the reaction mixture was cooled and diluted with EtOAc. After washing with brine, the organic phase was dried over Na_2SO_4 and the solvent removed to provide **54** (1.93 g, 85%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.59 (d, J = 7.8 Hz, 1H), 6.85 (s, 1H), 6.73 (m, 2H), 6.63 (d, J = 2.2 Hz, 1H), 6.57 (m, 2H), 6.06 (d, J = 2.2 Hz, 1H), 5.33 (s, 2H), 5.25 (s, 2H), 3.77 (br s, 1H), 3.39 (s, 3H), 3.38 (s, 3H), 2.90 (br s, 2H), 2.13–2.38 (m, 7H), 1.74 (m, 2H), 1.66 (m, 2H); LC–MS (ESI): m/z 513 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{26}H_{32}N_4O_7+H^+$ 513.5654, found 513.5651.

5.1.50. 5-{2-[4-(Dimethylamino)phenoxy]-4,6-dihydroxyphenyl}-*N*-(1-methylpiperidin-4-yl)-isoxazole-3-carboxamide (30)

To a stirred solution of **54** (512 mg, 1.0 mmol) and 37% HCHO solution (1.92 mL, 24 mmol) in a mixture of DCM/DMF (2:1, 5 mL) and AcOH (0.27 mL) was added portionwise tetramethylammonium triacetoxyborohydride (1.42 g, 5.4 mmol) at room temperature. After stirring for 1 h, the solution was diluted with EtOAc and thoroughly washed with saturated solution of sodium hydrogen carbonate. After drying over Na_2SO_4, the solvent was removed to afford 5-{2-[4-(dimethylamino)phenoxy]-4,6-bis(methoxymethoxy)phenyl}-*N*-(1-methylpiperidin-4-yl)-isoxazole-3-carboxamide, which was used directly in the next synthetic step. To a stirred solution of the crude in ethanol (2.5 mL) was added 4 N HCl in dioxane (2.5 mL). The reaction mixture was stirred overnight, evaporated to small volume and diluted with EtOAc. After washing with saturated solution of sodium hydrogen carbonate, the organic phase was dried over Na_2SO_4 and the solvent removed. The residue was purified by flash chromatography (eluent: DCM/MeOH/NH_3 aq 90:10:2) to provide **30** (230 mg, 51%, 2 steps). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.19 (br s, 1H), 9.80 (br s, 1H), 8.52 (d, J = 8.1 Hz, 1H), 6.90 (m, 2H), 6.77 (s, 1H), 6.74 (m, 2H), 6.14 (d, J = 2.2 Hz, 1H), 5.65 (d, J = 2.2 Hz, 1H), 3.72 (m, 1H), 2.87 (s, 6H), 2.75 (m, 2H), 2.15 (s, 3H), 1.91 (m, 2H), 1.70 (m, 2H), 1.61 (m, 2H); LC–MS (ESI): m/z 453 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{24}H_{28}N_4O_5+H^++H^+$ 453.2133, found 453.2131.

5.1.51. 5-{2,4-Dihydroxy-6-[4-(pyrrolidin-1-yl)phenoxy]phenyl}-*N*-(1-methylpiperidin-4-yl)-isoxazole-3-carboxamide (32)

To stirred solution of **54** (153 mg, 0.3 mmol), 2,5-dimethoxytetrahydrofuran (0.077 mL, 0.6 mmol) and 2.5 M sulfuric acid solution (0.3 mL, 0.75 mmol) in MeOH/tetrahydrofuran (1:1, 5 mL) at 5 °C was added portion wise sodium borohydride (46 mg, 1.2 mmol). After stirring overnight at room temperature, the solvent was removed and the residue taken up in EtOAc was washed with a saturated solution of sodium hydrogen carbonate. After drying over Na_2SO_4, the solvent was removed. The residue was purified by Biotage SP1 flash chromatography (gradient elution from 2% to 10% of MeOH in DCM) to afford 5-{2,4-bis(methoxymethoxy)-6-[4-(pyrrolidin-1-yl)phenoxy]phenyl}-*N*-(1-methylpiperidin-4-yl)-isoxazole-3-carboxamide (66 mg, 0.11 mmol), which was dissolved in ethanol (0.25 mL) and treated with 4 N HCl in dioxane (0.25 mL). The reaction mixture was stirred overnight, evaporated to small volume and diluted with EtOAc. After washing with saturated solution of sodium hydrogen carbonate, the organic phase was dried over Na_2SO_4 and the solvent removed. The residue was purified by flash chromatography (eluent: DCM/MeOH/NH_3 aq 80:20:2) to provide **32** (47 mg, 33%, 2 steps). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.51 (d, J = 8.1 Hz, 1H), 6.89 (m, 2H), 6.77 (s, 1H), 6.54 (m, 2H), 6.11 (d, J = 2.2 Hz, 1H), 5.61 (d, J = 2.2 Hz, 1H), 3.70 (m, 1H), 3.20 (m, 4H), 2.73 (m, 2H), 2.14 (s, 3H), 1.94 (m, 6H), 1.71 (m, 2H), 1.61 (m, 2H); LC–MS (ESI): m/z 479 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{26}H_{30}N_4O_5+H^+$ 479.2289, found 479.2284.

5.1.52. 5-{2,4-Dihydroxy-6-[4-(propan-2-ylamino)phenoxy]phenyl}-*N*-(1-methylpiperidin-4-yl)-isoxazole-3-carboxamide (31)

To a stirred solution of **54** (512 mg, 1.0 mmol) and acetone (0.147 mL, 2.0 mmol) in a mixture of DMF (5 mL) and AcOH (0.27 mL) was added portion wise tetramethylammonium triacetoxyborohydride (526 mg, 2.0 mmol) at room temperature. After stirring for 1 h, the solution was diluted with EtOAc and thoroughly washed with saturated solution of sodium hydrogen carbonate. After drying over Na_2SO_4, the solvent was removed to afford 5-{2,4-bis(methoxymethoxy)-6-[4-(propan-2-ylamino)phenoxy]phenyl}-*N*-(1-methylpiperidin-4-yl)-isoxazole-3-carboxamide, which was used directly in the next synthetic step. To a stirred solution of the crude in ethanol (2.5 mL) was added 4 N HCl in dioxane (2.5 mL). The reaction mixture was stirred overnight, evaporated to small volume and diluted with EtOAc. After washing with saturated solution of sodium hydrogen carbonate, the organic phase was dried over Na_2SO_4 and the solvent removed. The residue was purified by flash chromatography (eluent: DCM/MeOH/NH_3 aq 90:10:2) to provide **31** (303 mg, 65%, 2 steps). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.15 (s, 1H), 9.78 (s, 1H), 8.56 (d, J = 8.1 Hz, 2H), 6.77 (m, 2H), 6.76 (s, 1H), 6.56 (m, 2H), 6.11 (d, J = 2.2 Hz, 1H), 5.65 (d, J = 2.2 Hz, 1H), 5.30 (d, J = 7.9 Hz, 1H), 3.76 (m, 1H), 3.49 (m, 1H), 3.49 (m, 1H), 2.84 (m, 2H), 2.24 (br s, 3H), 1.76 (m, 2H), 1.65 (m, 2H), 1.12 (d, J = 6.2 Hz, 6H); LC–MS (ESI): m/z 467 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{25}H_{30}N_4O_5+H^+$ 467.2289, found 467.2289.

The following compounds **33**–**34** were prepared according to the method described above using the suitable ketone.

5.1.53. 5-{2-[4-(Cyclobutylamino)phenoxy]-4,6-dihydroxyphenyl}-*N*-(1-methylpiperidin-4-yl)-isoxazole-3-carboxamide (33)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.15 (br s, 1H), 9.78 (s, 1H), 8.53 (d, J = 8.1 Hz, 1H), 6.89 (d, J = 1.5 Hz, 1H), 6.80 (m, 2H), 6.76 (s, 1H), 6.51 (d, J = 8.9 Hz, 2H), 6.11 (d, J = 2.1 Hz, 1H), 5.81 (d, J = 6.9 Hz, 1H), 5.64 (d, J = 2.1 Hz, 1H), 3.78 (m, 1H), 3.71 (m, 1H), 2.77 (m, 1H), 2.32 (m, 2H), 2.17 (s, 3H), 1.90–2.03 (m, 2H), 1.76–1.85 (m, 2H), 1.41–1.74 (m, 6H); LC–MS (ESI): m/z 479 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{26}H_{30}N_4O_5$+H^+ 479.2289, found 479.2284.

5.1.54. 5-{2,4-Dihydroxy-6-[4-(tetrahydro-2*H*-pyran-4-ylamino)phenoxy]phenyl}-*N*-(1-methylpiperidin-4-yl)-isoxazole-3-carboxamide (34)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.15 (s, 1H), 9.78 (s, 1H), 8.56 (d, J = 8.1 Hz, 2H), 6.77 (m, 2H), 6.76 (s, 1H), 6.56 (m, 2H), 6.11 (d, J = 2.2 Hz, 1H), 5.65 (d, J = 2.2 Hz, 1H), 5.30 (d, J = 7.9 Hz, 1H), 3.89 (m, 2H), 3.83 (m, 2H), 3.76 (m, 1H), 3.66 (m, 1H), 2.84 (m, 2H), 1.90 (m, 2H), 1.76 (m, 2H), 1.65 (m, 2H), 1.47 (m, 2H), 1.12 (d, J = 6.2 Hz, 6H); LC–MS (ESI): m/z 509 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{27}H_{32}N_4O_6$+H^+ 509.2395, found 509.2395.

5.1.55. *tert*-Butyl 4-{[(5-{2-[4-(dimethylamino) phenoxy]-4,6-bis(methoxymethoxy)phenyl}-isoxazol-3-yl)carbonyl]amino}piperidine-1-carboxylate (55)

To a stirred solution of **52** (3.05 g, 4.85 mmol) in a mixture of dioxane/water (5:1, 33 mL) were added portion wise zinc powder (1.26 g, 19.4 mmol) and ammonium chloride (3.35 g, 48.5 mmol). After stirring at 100 °C for 3 h, the reaction mixture was cooled and diluted with EtOAc. After washing with brine, the organic phase was dried over Na_2SO_4 and the solvent removed to afford *tert*-butyl 4-[({5-[2-(4-aminophenoxy)-4,6-bis(methoxymethoxy)phenyl]-isoxazol-3-yl}carbonyl)amino]piperidine-1-carboxylate, which was used directly in the next synthetic step. To a stirred solution of the crude and 37% HCHO solution (9 mL, 120 mmol) in a mixture of DCM/DMF (2:1, 33 mL) and AcOH (2.8 mL) tetramethylammonium triacetoxyborohydride (6.9 g, 26.15 mmol) was added portionwise at room temperature. After stirring for 1 h, the solution was diluted with EtOAc and thoroughly washed with saturated solution of sodium hydrogen carbonate, and dried over Na_2SO_4. The solvent was removed and the residue was purified by flash chromatography (eluent: hexane/EtOAc 2:3) to afford **55** (2.28 g, 75%, 2 steps). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.67 (d, J = 8.1 Hz, 1H), 6.92 (m, 2H), 6.87 (s, 1H), 6.74 (m, 2H), 6.62 (d, J = 2.2 Hz, 1H), 6.06 (d, J = 2.2 Hz, 1H), 5.24 (s, 2H), 5.11 (s, 2H), 3.83–4.09 (m, 3H), 3.35 (s, 3H), 3.29 (br s, 2H), 3.33 (s, 3H), 2.88 (s, 6H), 2.81 (br s, 2H), 1.75 (m, 2H), 1.46 (m, 2H), 1.40 (s, 9H); LC–MS (ESI): m/z 627 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{32}H_{42}N_4O_9$+H^+ 627.3025, found 627.3021.

5.1.56. 5-{2-[4-(Dimethylamino)phenoxy]-4,6-dihydroxyphenyl}-*N*-(piperidin-4-yl)-isoxazole-3-carboxamide dihydrochloride (35)

To a stirred solution of **55** (2.0 g, 3.19 mmol) in ethanol (7.9 mL) was added 4 N HCl in dioxane (7.9 mL). The reaction mixture was stirred overnight, the solvent removed and the residue was triturated with diethyl ether and filtered to provide **35** (1.54 g, 95%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.40 (br s, 1H), 9.99 (br s, 1H), 8.84 (d, J = 7.7 Hz, 1H), 8.74 (d, J = 13.9 Hz, 2H), 8.48 (d, J = 10.0 Hz, 1H), 7.19 (br s, 2H), 7.01 (br s, 2H), 6.80 (s, 1H), 6.27 (br s, 1H), 5.76 (br s, 1H), 4.04 (m, 1H), 3.28 (m, 2H), 2.90–3.06 (m, 8H), 1.94 (m, 2H), 1.77 (m, 2H); LC–MS (ESI): m/z 439 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{23}H_{26}N_4O_5$+H^+ 439.1976, found 439.1975.

5.1.57. 5-{2-[4-(Dimethylamino)phenoxy]-4,6-dihydroxyphenyl}-*N*-[1-(4,4,4-trifluorobutyl)piperidin-4-yl]-isoxazole-3-carboxamide (36)

A solution of **35** (102 mg, 0.2 mmol), *N*,*N*-diisopropylethylamine (0.136 mL, 0.8 mmol) and 1-bromo-4,4,4-trifluorobutane (0.049 mL, 0.36 mmol) in DMF (3 mL) was stirred at room temperature overnight. The solution was taken up in EtOAc and thoroughly washed with brine, then dried over Na_2SO_4. After removal of the solvent, the residue was purified by flash chromatography (eluent: DCM/MeOH 90:10) to provide **36** (50 mg, 45% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.19 (s, 1H), 9.80 (s, 1H), 8.53 (d, J = 6.9 Hz, 1H), 6.90 (m, 2H), 6.77 (s, 1H), 6.74 (m, 2H), 6.14 (d, J = 2.2 Hz, 1H), 5.65 (d, J = 2.2 Hz, 1H), 3.74 (m, 1H), 2.87 (s, 6H), 2.84 (m, 2H), 2.19–2.38 (m, 6H), 1.97 (br s, 1H), 1.53–1.80 (m, 6H); LC–MS (ESI): m/z 549 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{27}H_{31}F_3N_4O_5$+H^+ 549.2320, found 549.2319.

5.1.58. *N*-(1-Cyclohexylpiperidin-4-yl)-5-{2-[4-(dimethylamino)phenoxy]-4,6-dihydroxyphenyl}-isoxazole-3-carboxamide (37)

To a stirred solution of **35** (102 mg, 0.2 mmol) and cyclohexanone (0.041 mL, 0.4 mmol) in DMF (3 mL) was added portion wise tetramethylammonium triacetoxyborohydride (105 mg, 0.4 mmol). After stirring at room temperature overnight, the solution was taken up in EtOAc and thoroughly washed with brine. After drying over Na_2SO_4, the solvent was removed and the residue was purified by flash chromatography (gradient elution from 10% to 20% of MeOH in DCM) to provide **37** (68 mg, 65%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.98 (s, 1H), 10.22 (s, 1H), 9.82 (s, 1H), 8.77 (br s, 1H), 6.90 (m, 2H), 6.78 (s, 1H), 6.74 (m, 2H), 6.15 (d, J = 2.2 Hz, 1H), 5.65 (d, J = 2.2 Hz, 1H), 3.95 (m, 1H), 2.87 (s, 6H), 1.53–2.03 (m, 9H), 0.90–1.42 (m, 6H); LC–MS (ESI): m/z 521 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{24}H_{26}N_4O_7$+H^+ 521.2759, found 521.2763.

The following compound **38** was prepared according to the method described above using the suitable ketone.

5.1.59. 5-{2-[4-(Dimethylamino)phenoxy]-4,6-dihydroxyphenyl}-*N*-[1-(1,4-dioxaspiro[4.5]dec-8-yl)piperidin-4-yl]-isoxazole-3-carboxamide (38)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.22 (s, 1H), 9.83 (s, 1H), 9.38 (br s, 1H), 8.88 (br s, 1H), 6.90 (m, 2H), 6.79 (s, 1H), 6.74 (m, 2H), 6.15 (d, J = 2.2 Hz, 1H), 5.65 (d, J = 2.2 Hz, 1H), 4.04 (br s, 1H), 3.87 (s, 4H), 3.06–3.50 (br s, 5H), 2.87 (s, 6H), 1.48–2.10 (m,12H); LC–MS (ESI): m/z 579 $[M+H]^+$; HRMS (ESI): m/z calcd for $C_{31}H_{38}N_4O_7$+H^+ 579.2814, found 579.2813.

5.2. NMR FAXS screening method

Fluorine NMR experiments were carried out at 564 MHz, using an Inova 600 instrument (Varian, Palo Alto, USA) equipped with a ^{19}F-^{1}H probe and with an autosampler. Samples for NMR screening were prepared in 50 mM Hepes buffer, pH 7.2, containing 100 mM KCl, 5 mM $MgCl_2$, 10 μM EDTA and 8% D_2O. A library of 300 fluorine-containing molecules was initially tested in mixtures (5–10 fluorine fragments in each mixture) against Hsp90 for the identification of potential spy molecules.[16] Spies are fluorine bearing (weak) ligands, whose displacement from a macromolecular target by a stronger ligand (screening compound) is reported by a fluorine signal intensification associated to the recovered unbound state of the spy molecule. Mixtures were tested at 50 μM concentration using ^{19}F one dimensional T_2 filter experiments recorded in the absence and presence of 1.5 μM Hsp90. As a result, 4-(3,4-dihydro-2*H*-1,5-benzodioxepin-7-yl)-6-(trifluoromethyl)pyrimidin-2-amine was identified as a suitable spy molecule. In addition, a molecule that does not interact with the receptor was selected from the library of 300 fluorine (CF and CF_3) compounds and used as

control molecule. Typical screening samples against Hsp90 contained 0.016 mg/mL (0.6 μM) Hsp90α (25,600 Da), 6 μM of the spy molecule (K_D 7.5 μM, value measured using a competition binding FP assay), 6 μM of control molecule, and 50 μM (0.2% DMSO-d_6) of the tested fragments. FAXS experiments were performed using the Carr–Purcell–Meibom–Gill (CPMG) spin-echo scheme[29] with length of 240 and 480 ms before the acquisition period. Spectra were collected at 293 K using 128 transients. Intensity reduction of the signal of the spy molecule in the presence of the protein due to shortening of its transverse relaxation is a marker of the interactions of the molecule with the protein. Intensity recovery of the signal in the presence of a competitive ligand that displaced the spy molecule is an indication of specific binding.

5.3. Crystallographic methods

Crystallization studies were performed using the N-terminal domain of Hsp90α (aa. 9-236). Crystals of the N-terminal domain of Hsp90 in complex with compounds **1**, **3**, **18** were grown from a solution of 20–25% MPEG 2000 K, 0.2 M $MgCl_2$, 0.1 M cacodylate pH 6.5–7.2 at 4 °C. The protein was concentrated at 25 mg/mL and compound was added to a nominal concentration of 2 mM. For data collection, the crystals were transferred to drops containing the equivalent mother liquor with 25% glycerol. Diffraction data for compounds **1** and **3** were collected in house using a Rigaku Micromax-007 HF X-ray generator and Mar345 Image Plate Detector (Marresearch) while data collection for compound **18** was carried out at the ESRF (Grenoble, France) on beamline ID23-2. Data were processed using the HKL package(Otwinowski, 1997).[30] Model building was done using Coot[31](Emsley,2004) and refinement was done with RefMac.[32](Murshudov, 1997) The coordinates have been deposited in the Protein Data Bank with code 4bqg, 4bqj and 4b7p for **1**, **3** and **18**, respectively, together with structure factors.

5.4. Hsp90. FP displacement assay

The FP assay was set up using as probe a reduced FITC-Geldanamycin (RFG) purchased from InvivoGen (San Diego, California). FITC-Geldanamycin was reduced as previously described[17] prior to use and then conserved in aliquots at −80°°C. In the experimental conditions used, the Z' value was higher than 0.6 and the CV of a standard control was of 23.9% (N = 57). For compounds Kd determination the mixtures containing Hsp90 and RFG at a final concentration of 5 and 0.5 nM, respectively were incubated for 3 h, and then compound serially diluted solutions in 100% DMSO were added to the mixtures at a final volume of 80 μL (final DMSO concentration 2%). The plate was incubated for 18 h at room temperature in the dark and then the FP signal was detected. Data were collected using a Tecan Safire2 reader, with excitation and emission wavelength at 470 and 525 nm, respectively, and then fitted using the program Dynafit version 3.28.039.[33]

5.5. In vitro pharmacology. A2780 cells proliferation assay

Cells were seeded into 96- or 384-wells plates at final concentration ranging from 10,000 to 30,000 cells per cm^2 in appropriate medium plus 10% FCS. After 24 h cells were treated using serial dilution of compounds in two replicates. At 72 h after the treatment the amount of cells were evaluated using the Cell Titer-Glo assay (Promega). Inhibitory activity was evaluated comparing treated versus control data using sigmoidal equation on the Assay Explorer (Symix) program.

5.6. Her2 degradation assay

Her2 degradation cellular activity of Hsp90 inhibitors was assessed by measuring the induced loss of Her2 protein levels in BT474 breast cancer cells. Cellular Her2 levels were measured by immunocytochemistry, and quantified using an ArrayScan vTi instrument (Cellomics Thermo Scientific). IC_{50} values represent the compound concentration at which cellular Her2 signal is diminished by 50% compared with untreated controls.

5.7. High throughput solubility

Solubility at pH 7 was performed as previously described.[34]

5.8. Hsc70 FP displacement assay

The FP specificity assay for Hsc70-FL was set up using a commercially available probe N^6-(6-amino)hexyl-ATP ATTO-590-ATP (JenaBioScience–Germany). For competition experiments, to the mixture containing Hsc70-FL and the fluorescent probe at a final concentrations of 600 and 20 nM, respectively, the appropriate compound solutions in 100% DMSO (2% final concentration) were added. The signal did not show any time dependency. Data were collected using an excitation and emission wavelength at 590 and 630 nm, respectively, and then analyzed as above described for Hsp90 FP displacement assay. For the Hsc70 FP assay the Z' value was higher than 0.8 and the CV of the standard control was 5.1% (N = 2).

5.9. Kinase assays

Interrogation for potential kinase inhibitory effects was performed on an internally developed Kinase Selectivity Screening (KSS) panel, designed to represent the overall diversity of the kinome, as described in detail in a recent publication.[35] The panel includes: ABL, ACK1, AKT1, Alk, AUR1, AUR2, BRK, CDC7, CDK2/CYCA, CHK1, CK2alpha/beta, eEF2K, EGFR1, ERK2, FAK, FGFR1, Flt3, GSK3beta, Haspin V473-K798, IGFR1, IKK2, IR, JAK1, JAK2, JAK3, KIT, LCK, MELK, MET, MK2, MPS1, MST4, NEK6, NIM, P38alpha, PAK4, PDGFRb, PDK1, PERK, PIM1, PIM2, PKAalpha, PKCbetaII, PLK1, RET, SULU1, SYK, TRKA, TYK2, VEGFR2, VEGFR3, ZAP70.

5.10. In vivo pharmacokinetics

The pharmacokinetic profiles of compounds were investigated in overnight fasted male Nu/Nu mice following a single dose given intravenously (iv). The vehicle used was 10% Tween 80 in 5% dextrose solution. A total of three mice were treated. Blood samples of each mouse were collected from the saphenous vein at predose, 0.083, 0.5, 1, 6, and 24 h postdosing. Samples were centrifuged at 10,000g for 3 min at 4 °C and the plasma was stored at −80 °C until analysis. Samples were analyzed by LC/MS/MS technique.

5.11. In vivo pharmacology

Evaluation of antitumor efficacy was performed as previously described.[34]

5.12. Cell based assays

The mechanism of action of the compounds was investigated using cell lines treated with compounds at the indicated concentration or with just DMSO as a control for 24 h. Client protein degradation was determined by Western blot analysis. Immunoblotting was done according to standard procedures and using the following antibodies: anti-Hsp70, anti-Hsp90 (Enzo Life Sciences, Farmingdale, NY), anti-ERK, anti-p-Thr202/204 ERK, anti-AKT, anti-pSer473 AKT. The SuperSignal chemiluminescence kit (Pierce) was used for detection.

Acknowledgments

We thank Mauro Paolucci for binding assays, Massimiliano Germani for PK/PD studies, the group of Assay Development and Biochemical Screening for the biochemical assay on a kinase panel, and the Experimental Therapy group for in vivo experiments.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.09.018.

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