New TRAP1 and Hsp90 chaperone inhibitors with cationic components: preliminary studies on mitochondrial targeting

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Abstract.

TRAP1 (Hsp75) is the mitochondrial paralog of the Hsp90 molecular chaperone family. Due to structural similarity among Hsp90 chaperones, a potential strategy to induce apoptosis through mitochondrial TRAP1 ATPase inhibition has been envisaged and a series of compounds has been developed by binding the simple pharmacophoric core of known Hsp90 inhibitors with various appendages bearing a permanent cationic head, or a basic group highly ionizable at physiologic pH. Cationic appendages were selected as vehicles to deliver drugs to mitochondria. Indeed, masses of new derivatives were evidenced to accumulate in the mitochondrial fraction from colon carcinoma cells and a compound in the series, with a guanidine appendage, demonstrated good activity in inhibiting recombinant TRAP1 ATPase and cell growth and in inducing apoptotic cell death in colon carcinoma cells.

TRAP1 (Tumor Necrosis Factor-Associated Protein 1, a mitochondrial paralog of Hsp90 chaperone family, also known as Hsp75) is a component of a mitochondrial pathway, selectively up-regulated in tumor cells and responsible for maintenance of mitochondrial integrity, thus favoring cell survival. Studies demonstrated that mitochondrial TRAP1, together with Hsp90, interacts with cyclophilin D (Cyp D), a regulator of permeability transition pore, and antagonizes the Cyp D-dependent apoptotic cascade, likely via a protein (re)folding mechanism[1]. In a context of cancer cells with TRAP1 overexpression, its

silencing was demonstrated to cause sudden growth inhibition and apoptosis, and this correlated with altered mitochondrial function and modified protein expression, thus suggesting that this pathway may represent a novel molecular target for anticancer therapy[2]. In this perspective, an attractive idea has been recently proposed regarding the delivery of TRAP1 inhibitors inside mitochondria, as a tool to conjugate anticancer activity together with selectivity toward cancer cells with high mitochondrial TRAP1 levels[3]. TRAP1 is also involved in protein homeostasis through an extramitochondrial quality control pathway involving the proteasome regulatory particle TBP7, and this function is relevant for TRAP1 antiapoptotic role[4-6]. Thus, several mechanisms are involved in multifaceted roles of TRAP1 in adaptive processes of cancer cells[7-10].

Several attempts have been made in recent years to increase the efficacy of anticancer therapy through a specific subcellular compartmentalization delivery of drugs. In this field, mitochondria have been considered an attractive target for their relevant metabolic roles altered in cancer models. An appropriate and specific drug-delivery system is required to design mitochondria-targeted drugs.[11-16] Indeed, many structures able to direct pharmacological compounds to mitochondria share the presence of a basic component or a permanent cationic lipophilic group, in order to cross over membranes by exploiting a very favorable electric gradient. For example, groups such as polyamines, protonated at physiological pH, have been successfully employed to carry and internalize biologically active compounds through mitochondrial membranes into the organelle[17]. These structures, indeed, not only allow mitochondrial membrane crossing, but also favor specific accumulation in the organelle. In the field of Hsp90 inhibitors, the so-called Gamitrinibs, a family of geldanamycin derivatives (17-AAG) linked to cyclic guanidines or triphenylphosphonium groups, have been proposed as a novel class of mitochondria-directed TRAP1/Hsp90 inhibitors[18,19,3]. Our study took advantage from the availability of a TRAP1 crystal structure recently provided by Sung and co-workers[20]. In fact, a further issue, besides the specific mitochondrial delivery, deals with the selectivity of TRAP1/Hsp90 ATPase inhibition. To this aim we searched for novel putative TRAP1 antagonists and, in this study, a number of derivatives, as compounds **1** and **2** in Figure 1, already known to be non- or partly ionizable Hsp90 inhibitors,[21] were selected since they were predicted to interfere also with TRAP1 activity, based on sequence homology between Hsp90 and TRAP1 structures. Furthermore, the Hsp90 inhibitors **1** and **2** were modified to enhance their accumulation into mitochondria.

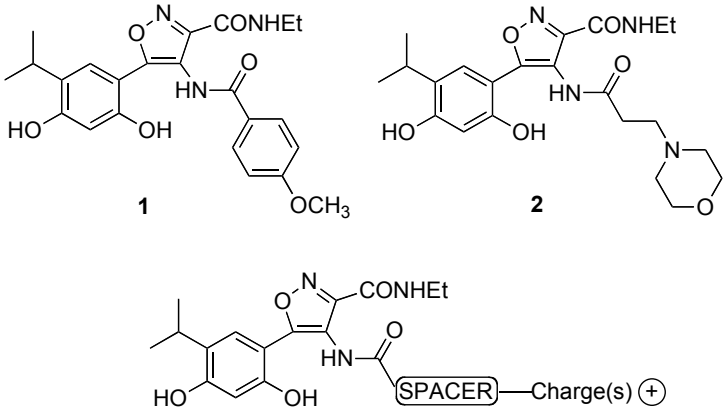


Figure 1. Known Hsp90 inhibitors and general structure of potential mitochondrial targeting derivatives.

The feasibility of the project was first investigated by preparing a small explorative sample of a three-portion structure so equipped:

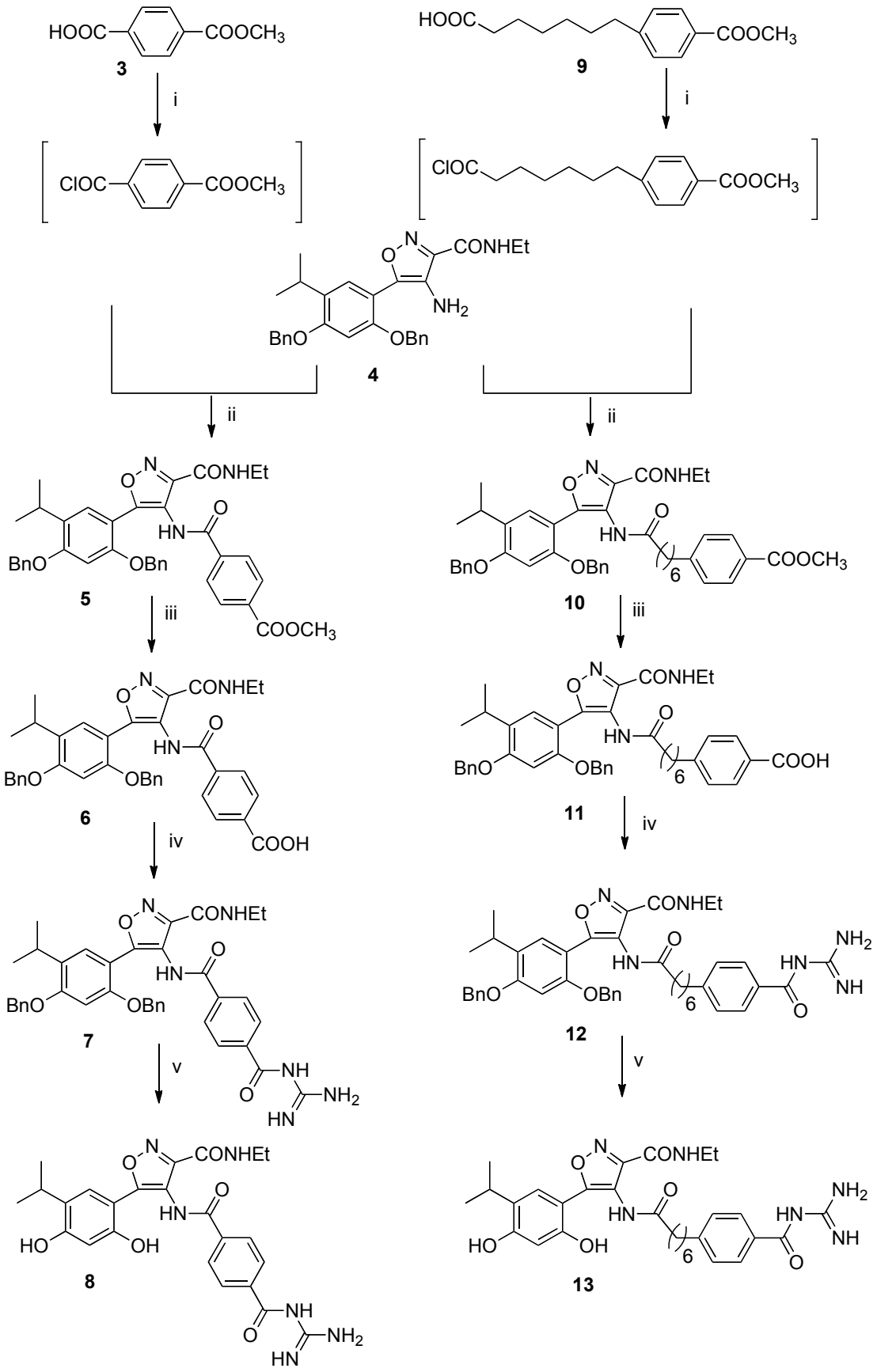
1) a common 3,4 isoxazole diamide structure, as seen in Figure 1, derived from known compounds active as Hsp90 inhibitors. The nitrogen at position 4 of isoxazole is particularly suitable to link a multitude of different appendages;

2) a cationic head, either as a permanent ion or as an ionizable group at physiologic pH;

3) a spacer between the portions above described, that can be chosen of various lengths.

In particular, a series of triphenylphosphonium as well as of pyridinium salts, and a guanidinium or a polyamine appendage have been considered as cationic heads to be linked to a common intermediate (**4**, Scheme 1), and were obtained as previously reported.[21.] Conjugate derivatives were comparatively evaluated by testing inhibition of recombinant TRAP1 ATPase activity, as well as accumulation in mitochondria, cell viability and induction of apoptosis in colorectal carcinoma cells.

The synthetic procedure is depicted in Schemes 1 and 2.

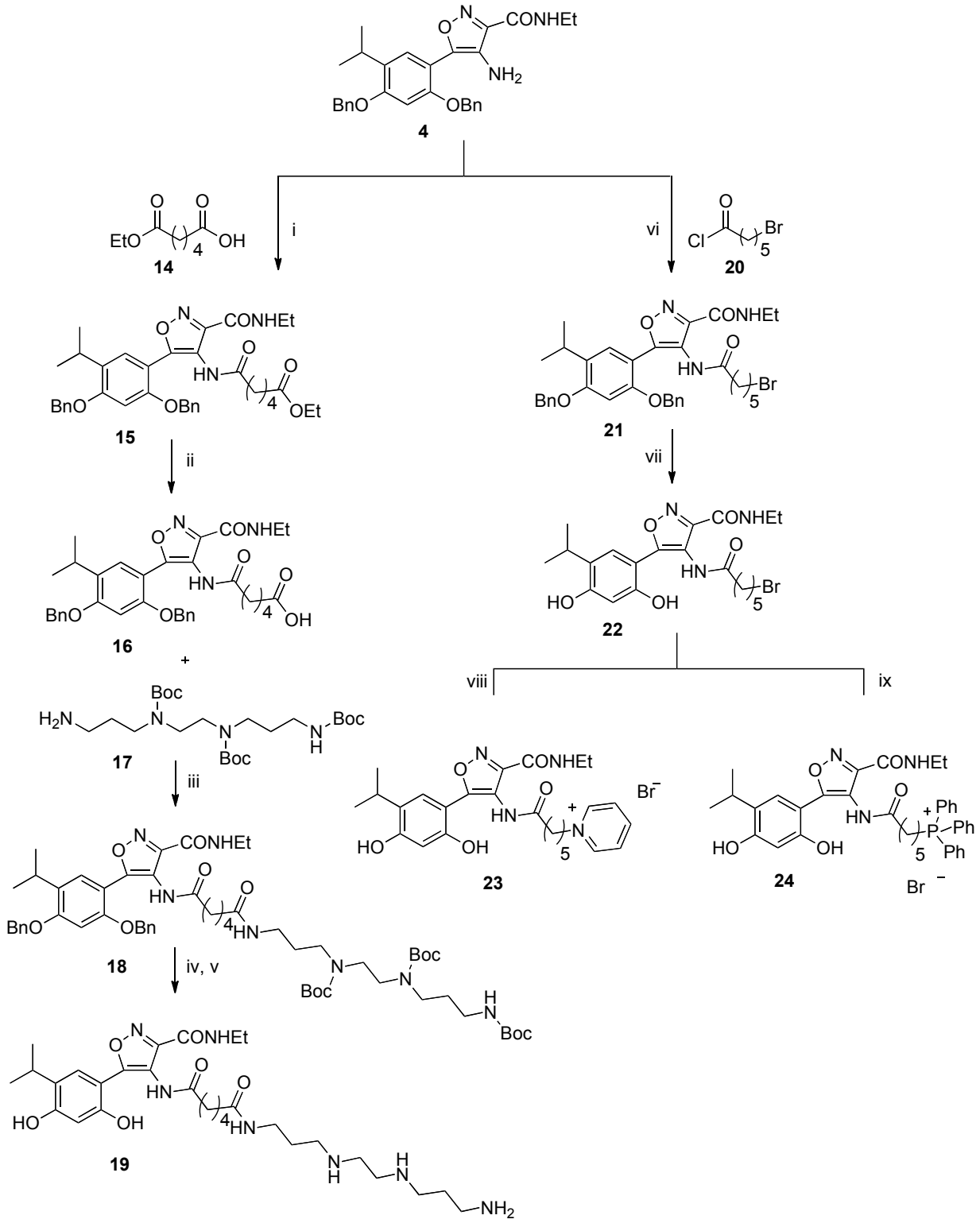


Scheme 1. Synthesis of guanidine bearing derivatives **8** and **13**. Reagents and conditions: i): oxalyl chloride, DMF, CH_2Cl_2, 4 h, rt; ii): CH_2Cl_2, TEA, 16 h, rt; iii): MeOH, 1N NaOH, H_2O, 24 h, 70 °C; iv): DMF, CDI, 1 h, rt, $CH_5N_3 \cdot HCl$, 16 h, rt; v): 1 M BCl_3, CH_2Cl_2, - 78 °C, 1 h.

The guanidine vehicle was introduced using two linkers of different length: the terephthalic acid monomethyl ester and the 7-(4-methoxycarbonylphenyl)-heptanoic acid were converted into acyl chlorides and reacted with intermediate **4**, obtaining compounds **5** and **10**, respectively. Ester hydrolysis and coupling with guanidine (obtained from the hydrochloride treated with potassium tert-butoxide) gave intermediates **7** and **12**, finally deprotected with BCl_3 to yield compounds **8** and **13**.

Similarly, compound **4** was reacted with hexanedioic acid chloride monoethyl ester to obtain compound **15**, then hydrolized and coupled with the protected amine **17** (obtained as described in literature[22]). The deprotection of compound **18** gave the final polyamine derivative **19**.

Finally, the permanent cationic derivatives were synthesized reacting compound **4** with 6-bromohexanoic acid chloride, then deprotecting **21** with BCl_3 and displacing bromine of **22** with either pyridine (as refluxing solvent) or triphenylphosphine in refluxing 1,4-dioxane to give compounds **23** and **24**, respectively.



Scheme 2. Synthesis of derivatives with polyamine (**19**), pyridinium (**23**) and phosphonium (**24**) appendages. Reagents and conditions: i): oxalyl chloride, DMF, CH_2Cl_2, 4 h, rt, TEA, CH_2Cl_2, 16 h, rt; ii): THF, LiOH · H_2O, 2 h, 70 °C; iii): CDI, THF, 24 h, rt; iv): TFA, CH_2Cl_2, 1 h, rt; v): 1 M BCl_3, CH_2Cl_2, - 78 °C, 1 h; vi): CH_2Cl_2, TEA, 12 h, rt; vii): CH_2Cl_2, 1 M BCl_3, - 78 °C; viii): Pyridine, 18 h, rf; ix): Ph_3P, 1,4-dioxane, 150 °C, 40 min.

All newly-synthetized compounds were tested for inhibition of recombinant TRAP1 ATPase activity and cell viability, accumulation in mitochondria, and induction of apoptosis in colon carcinoma HCT116 cells.

Based on the structural homology between TRAP1 and Hsp90, the newly synthesized compounds were tested in comparison with the isoxazole-amide **1**, recently emerged from our studies on Hsp90 inhibitors.[21] Two well-known potent Hsp90 inhibitors, i.e., AUY922 and Hsp990, obtained from Novartis, were also tested since they were also proposed as potentially acting on the TRAP1 ATPase domain.[23,24]

In preliminary experiments, the ability of the reported compounds to accumulate inside mitochondria was investigated. To this purpose, a mass spectrometric analysis on separated mitochondrial and cytosolic fractions purified from colorectal carcinoma HCT116 cells exposed to 1 μM of each agent for 12 h was used as a qualitative technology to assess the intracellular distribution of our compounds. We found molecular peaks corresponding to compounds **8**, **13**, **19** and **24,** but not to the reference compounds **1** and Hsp990, in the mitochondrial fractions of HCT116 cells. On the other hand, in the experimental conditions adopted, we did not find any of the tested compounds in the cytosolic compartment. Additional experiments are however necessary to quantitatively address the issue of the intracellular distribution of these compounds.

Data about ATPase activity inhibition of recombinant TRAP1 are reported in Table 1. The guanidine **8**, with an IC_{50} of 500 nM, showed an inhibitory activity comparable to that of the reference inhibitor **1** (IC_{50} = 400 nM)**,** as well as of Hsp990 and AUY922. The guanidine analogue with a longer linker **13** resulted six times less active, whereas a more substantial drop of activity was observed upon testing the polyamine **19** (5 μM), and the pyridinium **23** (10 μM). The phosphonium analogue **24** was almost inactive (25 μM), similarly to the reference inhibitor **2**.

Table 1. Compound IC_{50}s upon recombinant TRAP1 ATPase inhibition assay and MTT viability assay in HCT116 cells.

Compound	TRAP1 ATPase IC_{50} (μM)	MTT assay IC_{50} (μM)*
NVP-AUY922	0.5	Nd
Hsp990	0.2	Nd
1	0.4	0.87±0.08
2	20	Nd
8	0.5	1.32±0.11
13	3	6.17±0.56
19	5	26.7±2.1
23	10	7.86±1.04
24	25	5.12±0.68

*Nd: not done

Then we focused our attention on the possible dual activity of new compounds toward both TRAP1 and Hsp90 proteins and measured the affinities (K_d) of our best new TRAP1 inhibitor, compound **8**, towards TRAP1 and Hsp90. To this aim, we used compound **8** and geldanamycin-FITC (G-FITC) in a fluorometric competitive binding assay (see refs. 25 and 26 and the details in Supplementary data). First, we determined the dissociation equilibrium constants for the G-FITC/protein complexes using fluorescence anisotropy as the observable (results are shown in Figure S1 of the Supplementary data). We obtained K_ds 37 (±14) nM with Hsp90 and 48 (±20) nM with TRAP1. The first value is consistent with the 23 and 30 nM K_d previously reported for the geldanamycin/Hsp90 complex.[25,27] Thus, G-FITC has essentially the same affinity for the two proteins.

The decrease in the emission anisotropy of the FITC probe measured following increasing amounts of compound **8** to the G-FITC and either Hsp90 or TRAP1 protein solutions is shown in Figure 2. The anisotropy values calculated according to eq. 2 in the Supplementary data are shown as solid lines. The best fitting K_ds were 1.5 (± 0.5) and 10 (± 4) nM for the complexes of compound **8** with Hsp90 and TRAP1, respectively. The corresponding changes in standard free energy (T = 26 °C) are -50.5 (± 1.0) and -45.8 (± 1.2) kJ mol^{-1}. To compare our result for the **8**/Hsp90 complex with previous IC_{50} data obtained using a very similar fluorescence polarization approach with several other structurally closely related 3,4-isoxazolediamides, but lacking the guanidine appendage,[21] we recalculated the anisotropy r, using the above K_d value (1.5 nM), as a function of the concentration of the incoming ligand at the concentrations of G-FITC (5 nM) and Hsp90 (30 nM) employed in that work. The IC_{50} value thus obtained (see Figure S2 in the Supplementary data) is 21 nM and lies well within the range of IC_{50} values

reported for 29 substituted 3,4-isoxazolediamides. Overall, the K_d and $\Delta G°$ values found for the two complexes indicate similar affinities of compound **8** for Hsp90 and TRAP1, with only a slight preference for the former protein.

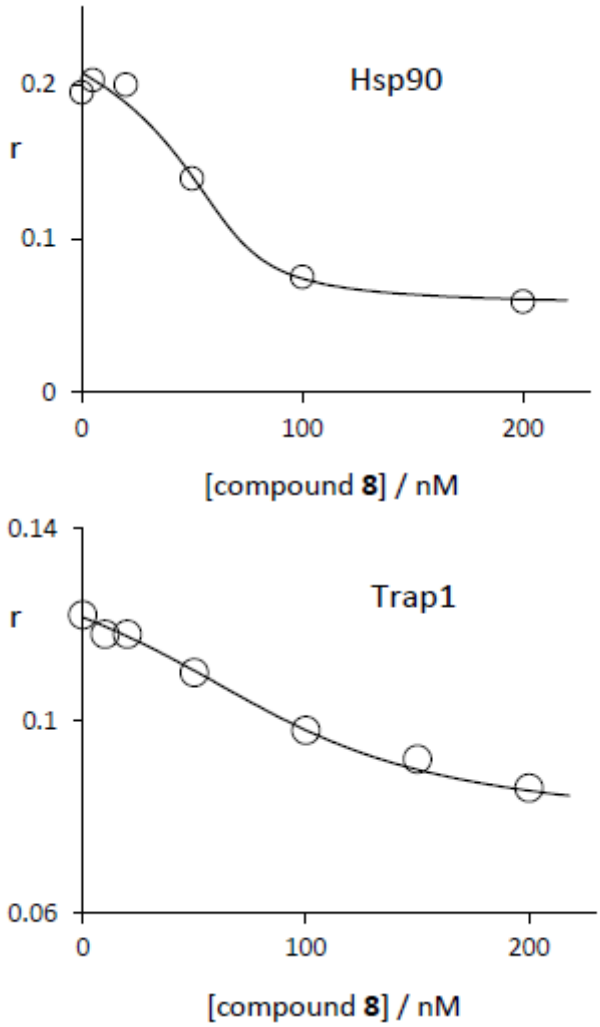


Figure 2. Emission anisotropy of the FITC probe in competitive binding experiments of compound **8** and geldanamycin-FITC (G-FITC) to proteins Hsp90 and TRAP1. Top: [Hsp90]=70 nM, [G-FITC]=12 nM. Bottom: [TRAP1]=100 nM, [G-FITC]=35 nM. λ_{exc}=470 nm, λ_{emiss}=525 nm. The solid curves represent anisotropies calculated according to eq. 2 in Supplementary data.

In subsequent experiments, all compounds were tested for their potential to inhibit cell proliferation, by MTT assay, upon 24 h exposure of colon carcinoma HCT116 cells to increasing concentrations of the drugs whose IC_{50} values are reported in Table 1. The MTT assay data showed that the guanidine **8** is active in the low micromolar range (IC_{50} 1.32 μM) with a cell growth inhibitory activity comparable to that of the reference **1** (IC_{50} 0.87μM), as previously demonstrated for other well-known mitochondria-delivered compounds[18]. By contrast, the phosphonium **24** (IC_{50} 5.12 μM), the guanidine analogue **13** (IC_{50} 6.17 μM), the pyridinium **23** (IC_{50} 7.86 μM) and the polyamine **19** (IC_{50} 26.7 μM) demonstrated lower or minimal activity.

Induction of apoptosis was evaluated in HCT116 cells upon 24 h treatment with increasing concentrations of reference and modified compounds (Figure 3). Compared to the reference compounds **1** and Hsp990 that are active starting from 0.1 μM, compound **8** showed cytotoxic activity starting from 1 μM. By contrast, compounds **23** and **24** induced apoptosis only in HCT116 cell exposed to 10 μM, polyamine **19** induced minimal and not dose-dependent apoptosis.

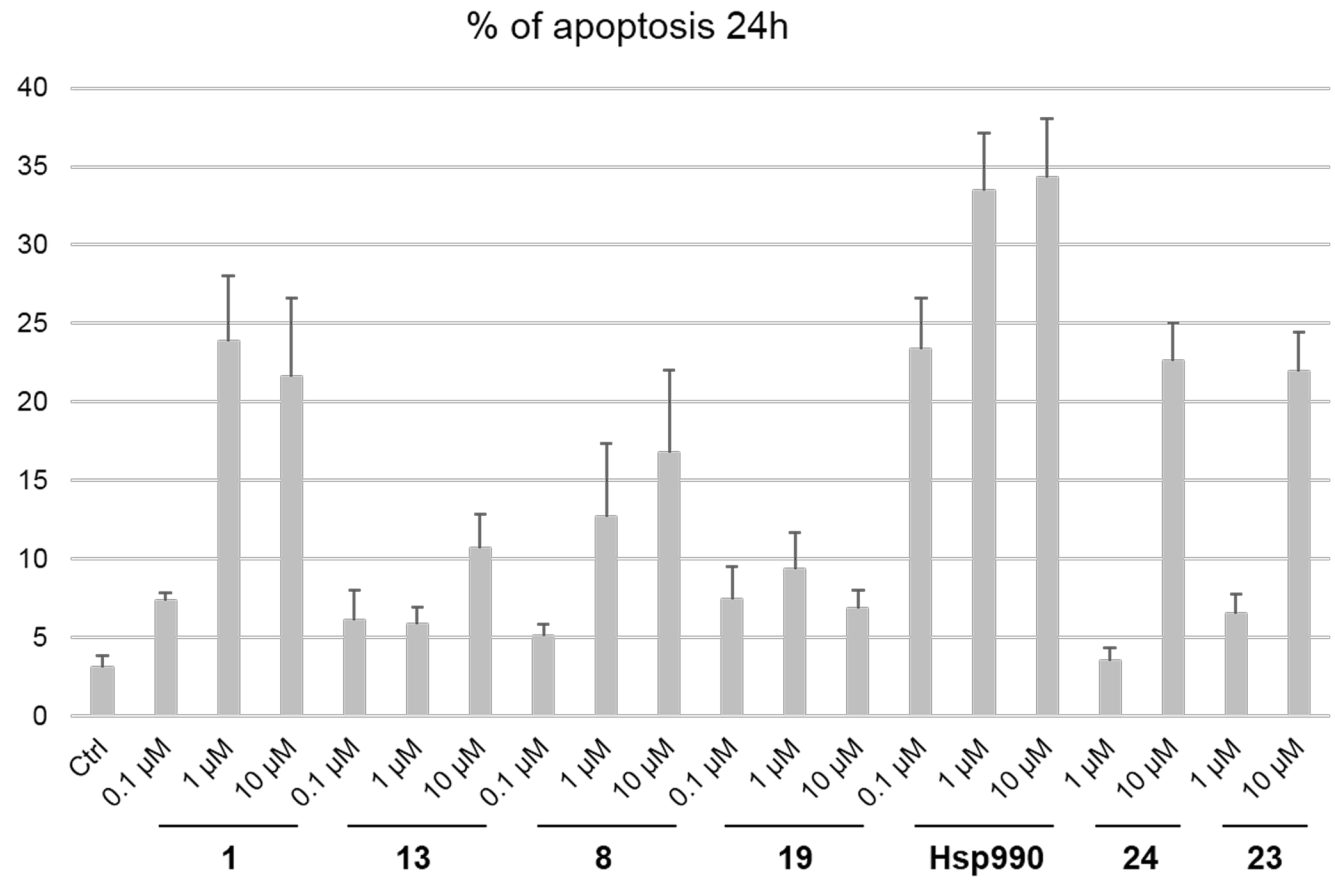


Figure 3. Percentage of apoptosis in HCT116 cells exposed for 24 hours to the indicated concentrations of new and reference compounds.

These results suggest that the isoxazole diamide scaffold, important to obtain active inhibitors of the Hsp90 chaperone family, can be easily modified in order to insert a cationic or a strongly basic group. These appendages are well known as mitochondrial targeting tools and are potentially useful to improve selectivity of therapeutic agents. We identified derivatized compounds in the mitochondrial fractions of tumor cells. Notably, compared to other known compounds[18], the guanidine **8** showed an interesting biological activity being able to inhibit TRAP1 ATPase in the nanomolar range and cell viability in the low micromolar range and to induce apoptosis. Despite sharing the same pharmacophoric core as **8**, other synthesized derivatives appear to be less active, both concerning cytotoxicity and TRAP1 ATPase inhibition. The guanidine **13**, with a longer linker than **8**, is the closest in the overall comparison of activities, being about six times less potent in both TRAP1 ATPase and MTT assays. Moreover, while the pyridinium **23** and the phosphonium analogue **24** have a similar cell proliferation IC_{50} as **13**, and a higher

apoptotic effect at 10 μM, their TRAP1 ATPase inhibition is lower. On the contrary, polyamine **19** demonstrated a negligible cell proliferation IC_{50} coupled with a fair ATPase activity. These findings suggest that other mechanisms, e.g. a poorer kinetics of cell membrane crossing, might influence their biological activity. Compound **8** was indeed our best identified inhibitor: it entered the mitochondria, while reference compound **1** did not, and maintained similar TRAP1 inhibitory potency. The recalculated IC_{50} value of 21 nM of this compound lies well within the values obtained for closely related 3,4-isoxazolediamides, but lacking the guanidine appendage and indicates that introduction of the latter did not produce any major change in the affinity for the Hsp90 protein. Compound **8** also featured high affinities for both Hsp90 and TRAP1 proteins, with K_ds 1.5 and 10 nM, respectively. The slightly higher affinity for the first protein corresponds to a ΔG° difference for compound/protein binding of 4.7 kJ mol^{-1}, only 10% of the ΔG° values. Future studies will address the issue of how increase the specificity of these novel mitochondria-directed agents toward TRAP1. Indeed, these novel compounds, besides having been selected based on the prediction to interfere with TRAP1 ATPase domain, are still dual Hsp90/TRAP1 inhibitors. Thus, in the perspective to design agents suitable for clinical use, it is mandatory that future inhibitors improve their selectivity toward single paralogs to enhance the anticancer activity and reduce the off-target toxicity. In this direction, the recent description of TRAP1 crystal structure [20] represents an important advancement for future drug design strategies.

In summary, these preliminary results showed that different appendages can be easily bound to a Hsp90 inhibitor core with the aim to obtain a mitochondrial drug accumulation. Initial biological results suggest that guanidine-based compounds are the most interesting potential hit structures, being able to interfere with TRAP1 function and to inhibit proliferation of colon carcinoma cells. However, further studies are needed to improve selectivity toward TRAP1.

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A. Supplementary data. Supplementary data associated with this article can be found, in the online version, at

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