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5-Aryl-4-(5-substituted-2,4-dihydroxyphenyl)-1,2,3-thiadiazoles as inhibitors of Hsp90 chaperone

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ABSTRACT

A series of 5-aryl-4-(5-substituted-2,4-dihydroxyphenyl)-1,2,3-thiadiazoles were synthesized and their binding to several constructs of human Hsp90 chaperone measured by isothermal titration calorimetry (ITC). The most potent compound bound Hsp90 with the dissociation constant of about 5 nM.

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Heat shock protein 90 (Hsp90), originally identified as one of several conserved heat shock proteins, exhibits general protective chaperone property—prevention of the unspecific aggregation of misfolded proteins.¹ Hsp90 constitutes about 1–2% of total cellular proteins. This protein is responsible for ATP-dependent folding, stability and functioning of many “client” proteins. Hsp90 functions are essential for development and progression of various cancers. Hsp90 inhibition leads to destabilization and degradation of many oncogenic proteins. Hsp90 is a promising anticancer drug target, as cancerous cells are more susceptible to Hsp90 inhibition than normal cells.^{2–7}

The ATPase activity of Hsp90 can be inhibited by natural products such as geldanamycin and radicicol (Fig. 1). Both of these compounds bind to the N-terminal domain of Hsp90 and inhibit the intrinsic ATPase activity.⁸ Geldanamycin showed activity in human tumor xenograft models but this compound proved to be too hepatotoxic for clinical development. However, the modified versions of geldanamycin, 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) and 17-demethoxy-17-[[2-(dimethylamino)ethyl]amino]geldanamycin (17-DMAG) retain the property of Hsp90 inhibition and have significantly less hepatotoxicity than geldanamycin.⁹ Phase 1 clinical trials of 17-AAG showed evidence of biological and clinical activities, including prolonged stable disease in two patients with melanoma.¹⁰ However, a second generation of Hsp90 inhibitors is being sought to overcome some of the undesirable features of

17-AAG, such as limited oral bioavailability, potential toxicity and poor aqueous solubility.^{11,12}

Radicicol is macrocyclic antibiotic isolated from *Monosporium bonorden*. Radicicol is more potent inhibitor of Hsp90 ATPase activity than geldanamycin or 17-AAG.¹³ Radicicol oximes have shown activity in animal models.¹⁴ However, no radicicol derivative has progressed to the clinic.

The first synthetic small molecule inhibitors of Hsp90 were based on the purine scaffold, for example, PU3 and PU24FCI (Fig. 1).¹⁵ Amongst other compounds, novobiocin and cisplatin have been reported to inhibit Hsp90 in these cases by binding at the C-terminal site.¹⁶

Various 3,4-diarylpyrazole¹⁷ (Fig. 1) as well as 4,5-diarylisoazole¹⁸ derivatives bearing resorcinol moiety have been selected by high throughput screening. These compounds showed high Hsp90 binding affinity and inhibitory effect on human cancerous cell line growth.¹⁹

Despite the fact that a large number of different Hsp90 inhibitors have been synthesized to date²⁰, only few of them are clinically tested. There still remains a great need of new potent Hsp90 inhibitors which offer one or more following advantages: improved activity, selectivity, solubility, reduced toxicity and side-effects, and reduced cost of synthesis.

Herein we report on a high-yielding synthesis of 5-aryl-4-(5-substituted-2,4-dihydroxyphenyl)-1,2,3-thiadiazoles and the results of in vitro binding to Hsp90 studies.

The chemistry employed for the design of the new compounds reported here is shown in Scheme 1. Synthesis of the starting

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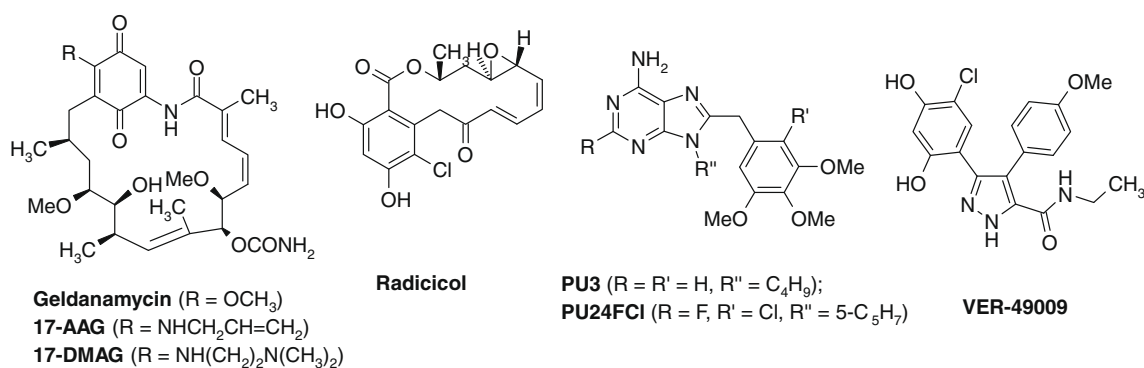
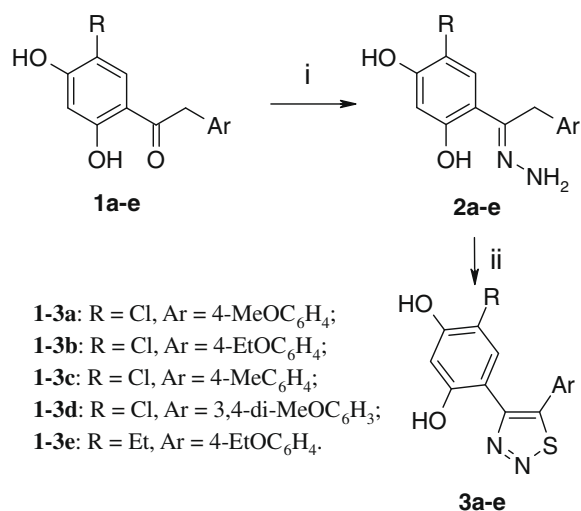


Figure 1. Schematic representation of some known Hsp90 binders.



Scheme 1. Reagents and conditions: (i) $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$ (2 equiv), EtOH, reflux, 7 h; (ii) SOCl_2 , rt, 2 h, then NaHCO_3 .

materials **1a–e** was accomplished as previously described.¹⁹ Compounds **1a–e** reacted with hydrazine hydrate in boiling ethanol and formed the corresponding hydrazones **2a–e**.²¹ Latter five derivatives underwent smoothly Hurd–Mori²² cyclization with thionyl chloride at room temperature to form 5-aryl-4-(5-substituted-2,4-dihydroxyphenyl)-1,2,3-thiadiazoles **3a–e** in high yields.^{23,24} It is noteworthy that Hurd–Mori reaction usually proceeded successfully when *N*-acyl- or *N*-tozylhydrazones bearing an adjacent methylene group were used. In our case, we observed smooth cyclization of unactivated hydrazones **2a–e**.

When compounds **2a** and **2e** were cyclized with thionyl chloride at reflux temperature, the formation of chlorinated side-products **4** and **5** together with **3a,e** was observed (Fig. 2).

The binding affinity of 5-aryl-4-(5-substituted-2,4-dihydroxyphenyl)-1,2,3-thiadiazoles to the full-length human Hsp90 protein

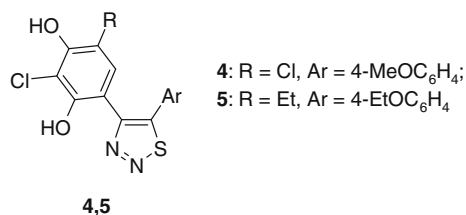


Figure 2. Structures of compounds **4** and **5**.

(Hsp90F) and the N-terminal domain of human Hsp90 (Hsp90N)²⁵ were determined by isothermal titration calorimetry (ITC).²⁶ Figure 3 shows representative isothermal titration calorimetry curves of **3b** binding to the Hsp90 N (50 mM Hepes buffer, 100 mM NaCl, pH 7.5, 37 °C). Protein concentration in the VP-ITC calorimeter (Microcal, Inc.) cell was 6 μM . Ligand concentration in the syringe was 120 μM . The binding constant was determined to be $1.6 \times 10^8 \text{ M}^{-1}$ with the stoichiometry of 0.97. This is equivalent to the dissociation constant of 6.3 nM. The steep transition of the ITC curve shows tight binding of 5-aryl-4-(5-substituted-2,4-dihydroxyphenyl)-1,2,3-thiadiazole to Hsp90 (see Fig. 4).

The strongest binder to both the Hsp90 N-terminal domain and the full-length Hsp90 was compound **3b** with the observed K_d of about 6.3 nM and 4.8 nM, respectively. Compounds **3a, c–e** also tightly bound to Hsp90. Compounds **4, 5** bearing chloro-substituent

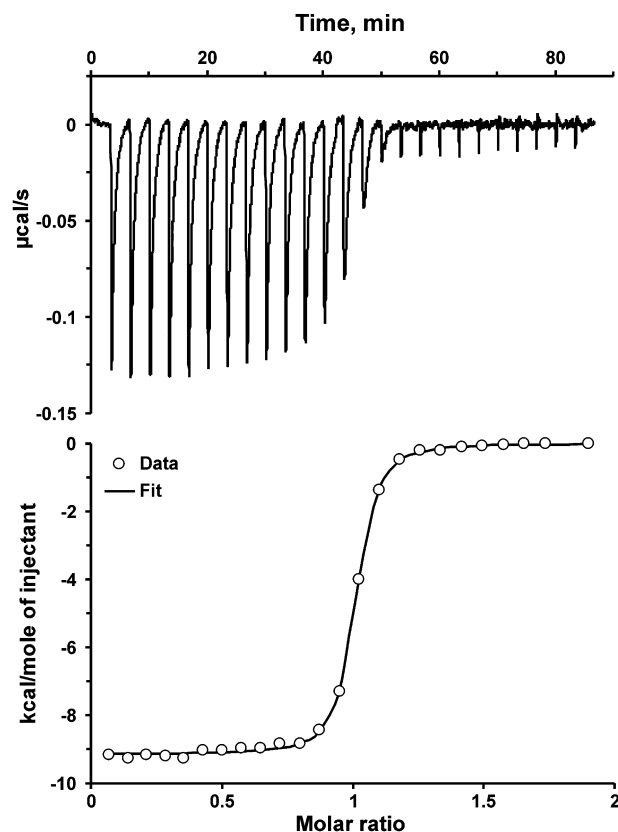


Figure 3. Isothermal titration calorimetry curve of **3b** binding to Hsp90N. The upper panel shows raw data and the lower panel-integrated data.

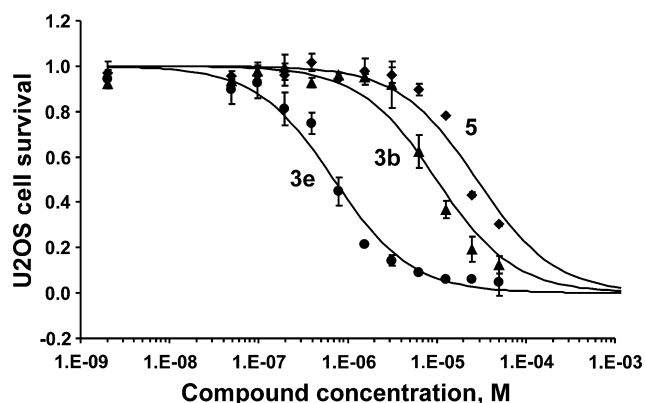


Figure 4. Normalized U2OS cancer cell line survival as a function of compound **3b**, **3e**, and **5** concentration.

Table 1

Dissociation constants of compounds **3a–e**, **4**, and **5** binding to both protein constructs (Hsp90N and Hsp90F) as determined by ITC at 37 °C

Compound	Hsp90N K_d μ M	Hsp90F K_d μ M
3a	0.013	0.0075
3b	0.0063	0.0048
3c	0.017	0.011
3d	0.034	0.039
3e	0.042	0.037
4	>20	>20
5	>20	>20
17-AAG	0.20	0.24

Values are means of multiple experiments.

in position 3 of the dihydroxyphenyl moiety practically did not bind to Hsp90. These compounds could not form the extensive *H*-bonding network involving both resorcinol hydroxyls due to the chloro-substituent, resulting in the lack of activity.¹⁹

The dissociation constants of compounds **3a–e**, **4**, and **5** with both protein constructs, obtained at 37 °C, are listed in Table 1.

Compound effect on cancer cells was tested by determining cell growth, death and survival as a function of compound concentration for two selected cancer cell lines, U2OS (osteosarcoma) and HeLa (cervical carcinoma)²⁷, using tetrazolium/formazan assay.²⁸ The strongest inhibitor of cancer cell growth was compound **3e** with the observed GI_{50} of 0.69 μ M for U2OS cells and 0.70 μ M for HeLa cells (Table 2, Fig. 4). Compound **5** was a relatively weak inhibitor of cancer cell lines. This property correlates well with its weak binding to Hsp90 (Table 2). Other compounds exhibited average potency of cancer cell growth inhibition. The compound series has potential to become candidates for therapeutic anticancer treatment.

In conclusion, a new group of compounds, similar to previously described diaryl pyrazoles¹⁹, was shown to be effective binders of

Table 2

U2OS and HeLa cancer cell line survival (growth inhibition, GI_{50}) by compounds **3a–e**, **5**

Compound	U2OS, GI_{50} , μ M	HeLa, GI_{50} , μ M
3a	6.0	2.5
3b	6.9	3.6
3c	7.1	3.3
3d	9.1	4.2
3e	0.69	0.70
5	28	19

Values are means of multiple experiments.

Hsp90 protein target and potent inhibitors of cancer cell survival and growth. A simple novel route has been employed for the compound synthesis.

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- General procedure for the preparation of 2-aryl-1-(5-substituted-2,4-dihydroxyphenyl)ethanone hydrazones (**2a–e**). Hydrazine hydrate (0.166 ml, 3.42 mmol) is added to a solution of the corresponding 2-aryl-1-(5-substituted-2,4-dihydroxyphenyl)ethanone **1a–e** (1.71 mmol) in 95% ethanol (5 ml). The mixture is heated under reflux for 7 h. Solvent is concentrated in vacuo, the residue treated with water, filtered off and recrystallized from 2-propanol. Spectral data for the selected compound: 1-(5-chloro-2,4-dihydroxyphenyl)-2-(4-ethoxyphenyl)-ethanone hydrazone (**2b**). Yield 97%, yellow solid, mp

- 163–165 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm 1.28 (3H, t, *J* = 6.6 Hz, CH₃), 3.94 (2H, q, *J* = 6.6 Hz, OCH₂), 3.94 (2H, s, CH₂), 6.39 (1H, s, ArH), 6.70 (2H, br s NH₂), 6.84 (2H, d, *J* = 8.7 Hz, ArH), 7.11 (2H, d, *J* = 8.7 Hz, ArH), 7.24 (1H, s, ArH), 10.18 (1H, br s OH), 13.55 (1H, s, OH). ¹³C NMR (75 MHz, DMSO-*d*₆) δ ppm 13.8, 28.6, 54.9, 104.1, 109.2, 113.0, 114.0 (2C), 127.2, 128.0, 129.1 (2C), 149.3, 153.2, 157.7, 158.4. Analysis: (C₁₆H₁₇ClN₂O₃) Calcd C 59.91%, H 5.34%, N 8.73%. Found: C 60.01%, H 5.45%, N 8.91%.
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23. *General procedure for the preparation of 4-(5-substituted-2,4-dihydroxyphenyl)-5-aryl-1,2,3-thiadiazoles (3a–e)*. The corresponding 2-aryl-1-(5-substituted-2,4-dihydroxyphenyl)ethanone hydrazone (**2a–e**) (0.33 mmol) is carefully added to thionyl chloride (1 ml). The reaction mixture is stirred at room temperature for 2 h. The excess of thionyl chloride is evaporated under reduced pressure, the residue is dissolved in chloroform (10 ml). The organic layer is washed twice with NaHCO₃ (sat. aq. 10 ml), then with water (15 ml), dried over Na₂SO₄, concentrated in vacuo. The residue was purified by column chromatography.
- Spectral data for the selected compound: 4-(5-chloro-2,4-dihydroxyphenyl)-5-(4-ethoxyphenyl)-1,2,3-thiadiazole (3b)*. Yield 80%, orange amorphous solid, mp 132–134 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm 1.30 (3H, t, *J* = 6.9 Hz, CH₃), 4.01 (2H, q, *J* = 6.9 Hz, OCH₂), 5.92 (1H, br s OH), 6.71 (1H, s, ArH), 6.93 (2H, d, *J* = 9 Hz, ArH), 7.24 (1H, s, ArH), 7.29 (2H, d, *J* = 9 Hz, ArH), 10.23 (1H, s, OH). ¹³C NMR (75 MHz, DMSO-*d*₆) δ ppm 15.2, 53.9, 104.7, 110.7, 111.1, 115.6 (2C), 120.8, 130.4 (2C), 132.2, 152.8, 154.2, 155.4, 156.0, 160.3. Analysis: (C₁₆H₁₃ClN₂O₅) Calcd C 55.09%, H 3.76%, N 8.03%. Found: C 55.19%, H 3.82%, N 8.14%.
24. Compounds **2a,c–e**, **3a,c–e**, **4** and **5** were also fully characterised by spectroscopic and elemental analysis data.
25. The gene encoding full-length human Hsp90α protein was purchased from RZPD, Deutsches Ressourcenzentrum für Genomforschung GmbH (Germany). For protein expression, full-length HSP90α sequence (Hsp90F) and the N-terminal fragment of the gene, corresponding to amino acids 3–241 (Hsp90N), were inserted into a multicloning site of pET-15b vector (Novagen, Madison, WI, USA). His₆-tagged proteins were expressed in the *Escherichia coli* strain BL21 (DE3). Hsp90F protein was purified using a Ni-IDA affinity column, followed by anion exchange chromatography on phosphocellulose p-11 and Q-sepharose (Amersham Biosciences). Hsp90N protein was purified on the Ni-IDA affinity column, followed by an anion exchange chromatography using DEAE-sepharose (Amersham Biosciences). Eluted proteins were dialyzed into a storage buffer containing 20 mM tris (pH 7.5), 50 mM Na₂SO₄, and 1 μM DTT, flash-frozen in liquid nitrogen and stored at –80 °C.
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27. HeLa cells (a kind gift from Dr. Aurelija Zvirbliene, Institute of Biotechnology, Lithuania) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, MO, USA), supplemented with 10% of fetal bovine serum (FBS) (Biocrom AG, Germany). U2OS cells were purchased from CLS–Cell Lines Service (Germany) and cultured in the medium composed of 50% DMEM and 50% of F-12 medium (Biocrom AG, Germany), supplemented with 10% FBS. Inhibitor stock solutions (20 mM) were made in 100% DMSO. Cells were seeded in 24-well culture plates and after 24 h subjected to different inhibitor concentrations (2-fold serial dilutions from 50 mM to 50 nM) and in the absence of inhibitor for control (with 0.25% DMSO in every well). After 72 h of incubation at 37 °C, 5% CO₂, inhibitor or only DMSO-containing culture medium in each well was replaced by 100 μl of Opti-MEM I medium (Invitrogen, CA, USA), containing 0.2 mg/ml XTT (sodium salt of 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2 H-tetrazolium-5-carboxyanilide inner salt) (Invitrogen, CA, USA) and 0.002 mg/ml PMS (phenazine methosulfate) (Sigma, MO, USA). After 20 min, solution from each well was taken out for spectrophotometric measurement of OD₄₇₀ to determine the relative amount of soluble XTT formazan in each well. All experiments were performed at least in duplicates.
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