REVIEW

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Binding of Natural and Synthetic Inhibitors to Human Heat Shock Protein 90 and Their Clinical Application

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Key words: *Hsp90 inhibitors; anticancer activity; isothermal titration calorimetry; thermal shift assay; ThermoFluor®.*

Summary. *This review describes the recent progress in the field of heat shock protein 90 (Hsp90) inhibitor design. Hsp90 is a heat shock protein with a molecular weight of approximately 90 kDa. Hsp90 is considered a good anticancer target because its inhibition leads to inactivation of its numerous client proteins participating in various signaling and other processes involved in cancer progression. Numerous Hsp90 inhibitors-leads currently tested in clinical trials are presented in this review.*

Furthermore, this review emphasizes the application of biophysical binding assays in the development of Hsp90 inhibitors. The binding of designed lead compounds to various Hsp90 constructs is measured by isothermal titration calorimetry and thermal shift assay. These assays provide a detailed energetic insight of the binding reaction, including the enthalpy, entropy, heat capacity, and the Gibbs free energy. A detailed description of the binding energetics helps to extend our knowledge of structure-activity relationships in the design of more potent inhibitors. The most active compounds are then tested for their absorption, distribution, metabolism, elimination, toxicity, and activity against cancer cell lines.

Introduction

Heat shock protein 90 (Hsp90) is a highly conserved molecular chaperone that plays an important role in protein regulation in cells. It accounts for nearly 1% of the total protein of the cell and is involved in the folding of many proteins, maintains their stability, protects from aggregation, and is a component of cellular machinery (1–3). Hsp90 was found to be overexpressed in a wide range of tumors, and thus it became a target of interest in oncology. Selectivity of natural and synthetic Hsp90 inhibitors toward cancer cells was demonstrated; thus, they are being developed as anticancer drugs (4–6). Currently, approximately 15 drug candidates are being tested as single agents or combined with other anticancer drugs in phase 1, 2, or 3 clinical trials.

Some of Hsp90 inhibitors bind to the N-terminal domain at the active site of ATP-binding pocket while others bind to the C-terminal domain. There are two groups of inhibitors binding at the N-terminal domain designed based on natural compounds: geldanamycin and radicicol. Both the compounds have been modified into new derivatives with desired efficiency and reduced toxicity (7, 8). Geldanamycin has been modified to 17-AAG and 17-DMAG, while various resorcinol-bearing compounds were designed based on radicicol. Here we describe the thermodynamics of their binding to Hsp90 by isothermal titration calorimetry (ITC) and thermal shift assay (TSA). These assays together with structural information of the Hsp90-inhibitor complex provide insight into the structure-activity relationship (SAR) of the compounds. The SAR helps in the process of rational drug design (9).

Hsp90 Structure

The molecular weight of Hsp90 is approximately 90 kDa, and it is found in all prokaryotic and eukaryotic cells. There are two highly homologous isoforms in human: α and β. Alpha isoform is prevalent (10), and there are no major known functional differences between the isoforms. Hsp90 homolog in yeast is named Hsc82 and also shares significant homology with human isoforms. Prokaryotic Hsp90 is called HtpG (for high temperature protein G), and it lacks the charged linker between the N-terminal and the middle domains (11). *Plasmodium falciparum* Hsp90 is 64% identical to its human analog (12). As shown in the sequence comparison of Hsp90 homologs in distant kingdoms (Fig. 1), Hsp90 is a highly conserved protein (13, 14).

The structure of yeast Hsc82 is shown in Fig. 2. The protein consists of three major domains: the amino terminal (N) domain (1–216 amino acids), the middle (M) domain (262–524 amino acids), and the carboxy terminal (C) domain (525–709 amino

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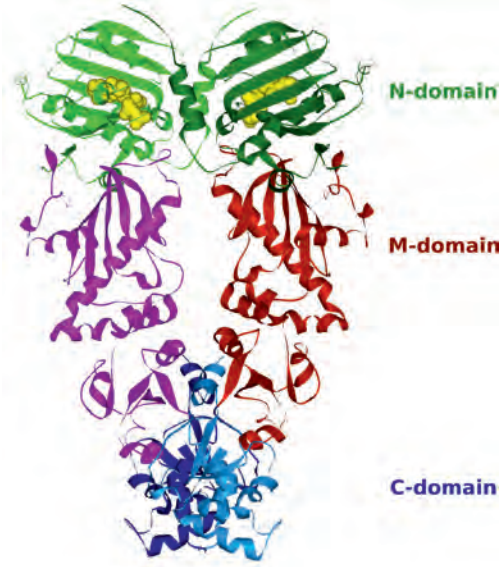
Fig. 1. Alignment of amino acid sequences

1, human Hsp90 alpha; 2, human Hsp90 beta; 3, *Plasmodium falciparum* Hsp90; 4, *Saccharomyces cerevisiae* Hsc82; and 5, *Helicobacter felis* HtpG. Amino acid residues with high consensus (90%) are shown in red, amino acids with 50% consensus are shown in blue. Different regions of the protein are boxed by different colors: ☐, N-terminal domain; ☐, M-domain; and ☐, C-terminal domain.



acids) (3). There is a charged linker between N-terminal and middle domains, but so far it was not possible to obtain the crystal structure of this region, and thus its structure is unknown (15).

The function of Hsp90 has been studied and reviewed in a number of manuscripts (2, 3). ATPase activity is necessary for the chaperone cycle, and inhibitor binding in the N-terminal domain ATP-binding pocket blocks the activity of Hsp90 and formation of its complex. Inhibition leads to client protein degradation and cell death (16).

Fig. 2. The structure of full-length Hsc82 dimer (PDB ID: 2cg9)

One monomer is shown in dark green-red-dark blue and another in light green-pink-light blue. Green shows the position of the N-terminal domains. ATP is bound in the N-terminal domain and is shown in yellow.

Inhibitors of Hsp90

The majority of Hsp90 inhibitors bind to the N-terminal ATP pocket and block ATPase activity (Fig. 3). Hsp90 inhibition was thoroughly studied based on geldanamycin binding to Hsp90 and discussed in (17, 18). This natural compound from ansamycin family was found to be a strong inhibitor of chaperone in vitro and in vivo, but it demonstrated undesired liver toxicity (19) and could not be developed further as an anticancer agent. Later, some of its derivatives, namely 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-(2-dimethylaminoethyl)amino-17-demethoxygeldanamycin (17-DMAG), have been developed and showed rather good antitumor activity (20). 17-AAG was the first Hsp90 inhibitor studied in clinical trials. Despite its high potency, 17-AAG showed poor solubility and stability and demonstrated moderate toxicity in several clinical trials (21). This compound attracted more attention after it was revealed as a useful agent for combined therapy since it enhanced efficacy of other chemotherapeutic agents (22).

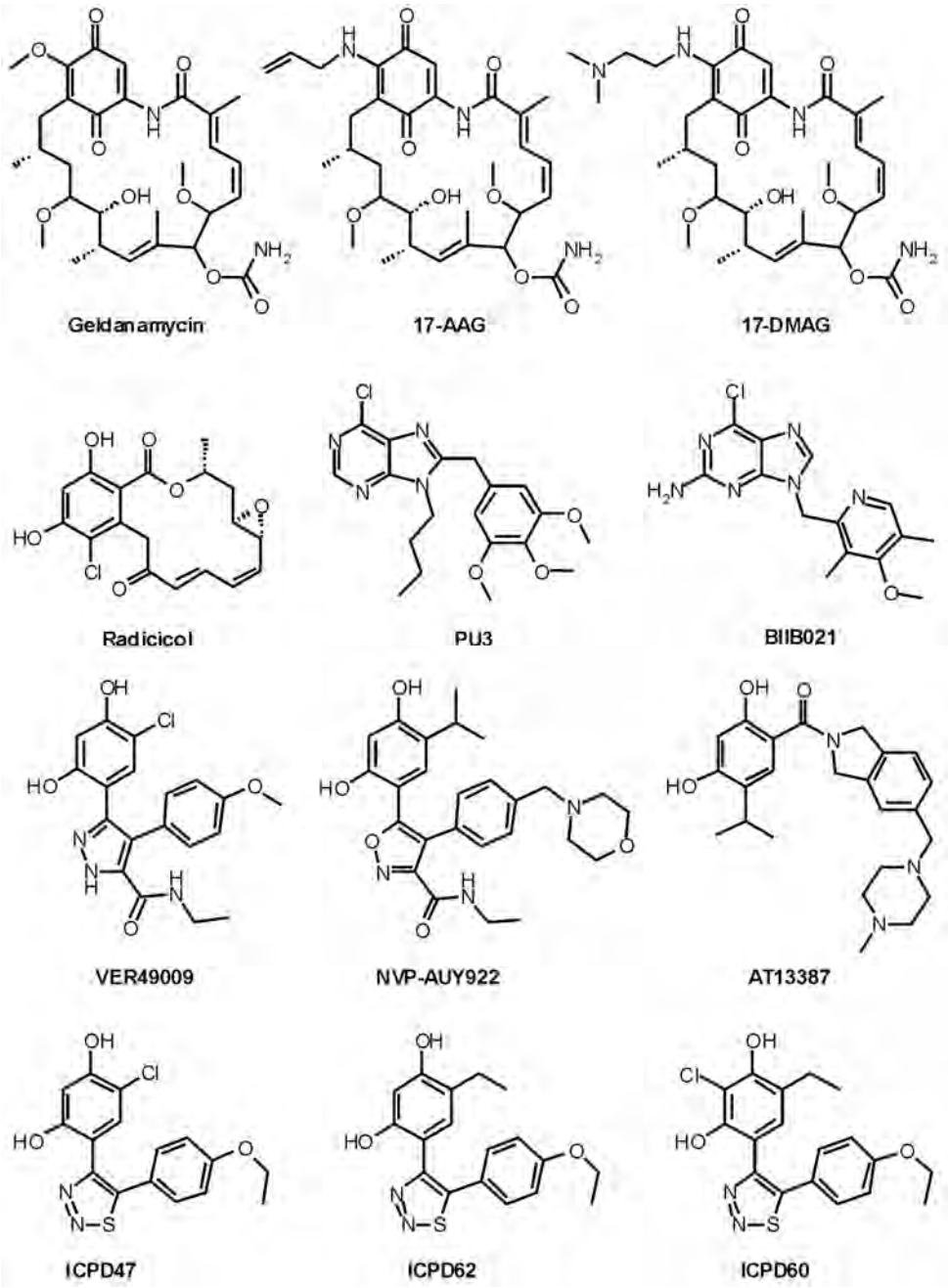


Fig. 3. The chemical structures of selected Hsp90 inhibitors described in this review

Thermodynamics of binding of ICPD inhibitors based on resorcinol scaffold is described below.

Another natural product, radicicol, is a very strong Hsp90 inhibitor and competes with ATP binding in the N-terminal domain. Despite its high activity in vitro, radicicol lacks in vivo efficacy. However, its resorcinol-bearing derivatives possess good antitumor activity in animals (23).

Experience with the natural products encouraged scientists to seek for new synthetic Hsp90 inhibitors. Based on ATP structure, a group of purine derivatives has been designed (24, 25). One of them (PU3) demonstrated better solubility than clinical candidate 17-AAG but was not as potent. After some optimizations, better inhibitors have been found (26). Compound BIIB021 was identified to be a stronger inhibitor in vitro and in vivo as compared with 17-AAG (27) and is now being tested in phase 2 clinical trials.

Using structure-based design approach, diarylpyrazole derivatives have been identified as potent Hsp90 inhibitors (28). VER49009 was the most active, and its binding mode to the N-terminal domain was studied using x-ray crystallography. Water molecules, essential for binding, were identified, and the deeper understanding of binding features led to the identification of an Hsp90 inhibitor, NVP-AUY922, which was the second synthetic compound to enter clinical trials and demonstrate early success (29, 30).

Fragment-based drug discovery approach, focusing on physicochemical and pharmacokinetic properties, yielded the compound AT-13387. It showed not only good efficacy and selectivity, but also high solubility and metabolic stability; therefore, it was selected for clinical development (31).

Several additional groups of Hsp90 inhibitors have been discovered and successfully entered clinical trials. According to the US National Institutes of Health website (www.clinicaltrials.gov), at least 15 Hsp90 inhibitors as single agents or in combinations are being investigated in clinical trials (Table), and more than 40 clinical trials have been already completed (32). New inhibitors have been designed based on benzamide (33), 2-aminothieno[2,3-d]pyrimidine (34), and dihydroxyphenylisoindoline (35) scaffolds. There are at least several classes of compounds that have not been disclosed yet.

Most Hsp90 inhibitors are investigated as anticancer agents for a wide range of different types of tumors (36, 37). However, there are also data supporting their application for the treatment of neurodegenerative diseases, such as Alzheimer's disease (38), Parkinson's disease, multiple sclerosis, Huntington's disease (39), rheumatoid arthritis (40), diabetes, ischemia (41), systemic lupus erythematosus (42), cystic fibrosis (43), fungal bacterial protozoan and viral infectious diseases (12, 44–46) and even are used as male contraceptives (47).

The most advanced drug candidates are 17-AAG that has already passed phase 3 clinical trials in combination with bortezomib in patients with relapsed-refractory multiple myeloma and STA-9090 that is currently being tested in phase 3 clinical trials in patients with advanced non–small lung cancer. Five compounds are being tested in phase 1 or 2 clinical trials and are promising leads in treating patients with Hsp90 inhibitors. However, during the last few years, some clinical trials with compounds XL888, IPI-493, and ABI010 have been terminated because of sponsor's decision, discontinuation of program, or for example, due to the fact that drug exposure of retaspimycin HCl was found to be superior to IPI-493, and Infinity Pharmaceuticals, Inc., has decided to focus exclusively on retaspimycin.

Table. Hsp90 Inhibitors in Clinical Trials

Inhibitor	Combined Drug	Phase	Route
Tanespimycin (17-AAG)	Alone or with bortezomib or gemcitabine hydrochloride	1/2/3	Intravenous
Retaspimicyn (IPI-504)	–	1/2	Intravenous
Alvespimycin hydrochloride (17-DMAG)	Alone or with trastuzumab or paclitaxel	1	Intravenous
NVP-AUY922	Alone or combined with erlotinib hydrochloride, capecitabine, trastuzumab or cetuximab	1/2	Intravenous
HSP990	–	1	Oral
AT13387	Alone or with imatinib	1/2	Intravenous
BIIB021	–	1/2	Oral
BIIB028	–	1	Oral
Debio 0932	–	1	Oral
DS-2248		1	Oral
KW-2478	Bortezomib	1/2	Intravenous
MPC-3100	–	1	Oral
PU-H71	–	1	Intravenous
SNX-5422 mesylate	–	1	Oral
STA-9090	Alone or with docetaxel	1/2/3	Intravenous

Thermodynamics of Hsp90 Ligand Binding

Detailed knowledge about structure-activity relationships could help in the development of new and more potent inhibitors (48). However, the thermodynamic characterization of ligand binding to Hsp90 chaperone is rather fragmented despite its importance. Here we describe some basics of protein-ligand binding thermodynamics.

Protein-ligand binding equilibrium is described by the Gibbs free energy of binding ($\Delta_b G$). More negative $\Delta_b G$ indicates a stronger binding reaction. However, several thermodynamic parameters that contribute to the $\Delta_b G$ can be correlated with the structural features of the protein-ligand complex easier than the $\Delta_b G$ itself. The most important parameters are the enthalpy ($\Delta_b H$) and the entropy ($\Delta_b S$) of binding:

$$\Delta_b G = \Delta_b H - T\Delta_b S \quad (1).$$

Both the enthalpy and entropy are the first temperature derivatives (T-derivatives) of the Gibbs free energy:

$$\left(\frac{\partial \Delta_b G}{\partial T}\right)_P = -\Delta_b S \quad (2);$$

$$\frac{\partial \ln K_b}{\partial T} = \frac{\Delta_b H}{RT^2} \quad (3).$$

The second T-derivative of the $\Delta_b G$ is the heat capacity of binding ($\Delta_b C_P$). Subscript P indicates constant pressure.

$$\Delta_b C_p = \left(\frac{\partial \Delta_b H}{\partial T}\right)_p \quad (4).$$

A number of various methods may be used for the measurement of the Gibbs free energy of ligand binding (49). Here we concentrate on two methods, ITC and TSA, also known as differential scanning fluorimetry (50). Both the methods have been previously reviewed, but ITC is used more widely (51, 52) than TSA despite its some important advantages (53–56).

ITC directly measures the heat evolved or absorbed during the binding reaction. This method is the most robust and accurate way of measuring the $\Delta_b H$. However, the ITC has a number of disadvantages. Most importantly, the binding constant should be in a rather narrow range to satisfy the requirement that coefficient c would be between about 5 and 500. The c is calculated as follows:

$$c = nM_t K_b \quad (5),$$

where n is the binding stoichiometry, M_t is the protein molar concentration, and K_b is the binding constant defined for the reaction of $M + L \leftrightarrow ML$ as:

$$K_b = \frac{[ML]}{[L][M]} \quad (6).$$

K_b is related to the Gibbs free energy:

$$\Delta G = -RT \ln K_b \quad (7).$$

In practice, ITC is useful for K_bs in the range of 10^5 to 10^9 M^{-1}. Another disadvantage of ITC is that it requires rather a large amount of protein (usually more than 0.1 mg) and ligand. These disadvantages can be quite easily approached using TSA. This method is based on the observation that specifically binding ligands stabilize (sometimes destabilize) the protein. TSA requires only several micrograms of protein. Furthermore, there is no upper limit of the K_b to be determined. The only limit is the temperature of water boiling. Therefore, extremely tight reactions as radicicol binding to Hsp90 can be studied by TSA. However, TSA does not determine D_bH, D_bS, and D_bC_p as distinct from ITC. Therefore, both ITC and TSA could be used together for an increased precision of the measurements (56).

Fig. 4 shows a typical ITC binding curve of the Hsp90-ligand system. Due the linked protonation, it is important to dissect protonation thermodynamics from binding thermodynamics in order to determine the intrinsic thermodynamics of binding. For example, the enthalpy of TRIS buffer protonation is so large (about –44 kJ/mol) that it would significantly alter any binding enthalpies. Therefore, a series of experiments in various buffers are necessary (55).

The binding of measurement by TSA is shown in Fig. 5. Panel A shows typical raw protein melting curves at various ICPD47 concentrations added. Fig. 5B shows the T_m shift curves for 17-AAG and ICPD47 as a function of inhibitor concentration. ICPD47, a more potent binder than 17-AAG, shifts the temperature by up to 12°C, while 17-AAG, a weaker binder, shifts the temperature by up to 4°C.

True intrinsic binding thermodynamic parameters could be obtained only after the detailed proton linkage and temperature analysis as previously described (55). This analysis and the crystallographic structure of Hsp90-inhibitor complex are not the subject of this review but are in preparation for publishing (unpublished data). The intrinsic dissociation constants were 1.1 nM for ICPD47 and 2.0 nM for ICPD62. However, the ICPD60 binding could not be detected, thus its K_d is weaker than 200 μM.

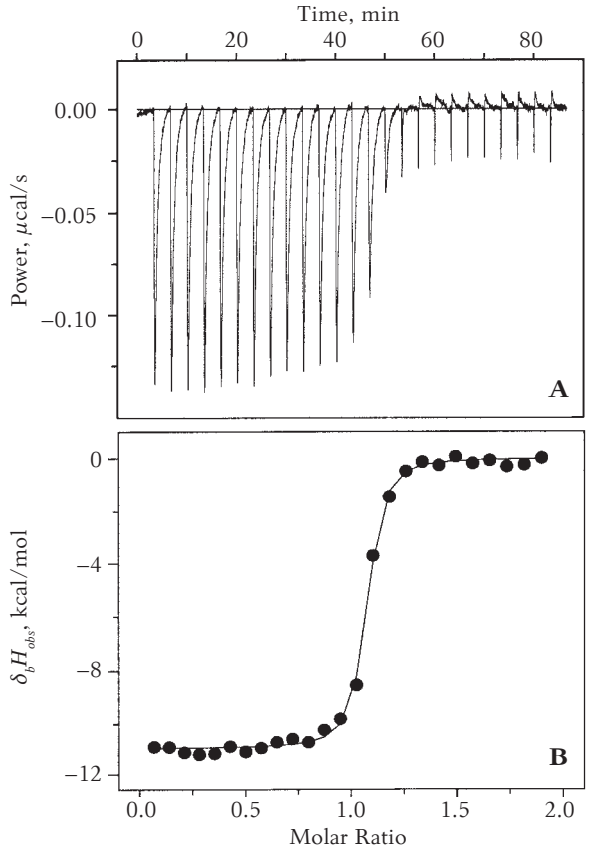


Fig. 4. Isothermal titration calorimetry data for ICPD47 binding to Hsp90αN

Panel A, raw ITC data; panel B, integrated ITC data with the curve fit to the standard single binding site model. The cell contained 4 μM protein, while the syringe contained 40 μM ICPD47 in the same buffer – 60 mM sodium phosphate, pH 7.5, 0.5% DMSO, 100 mM NaCl, at 37°C.

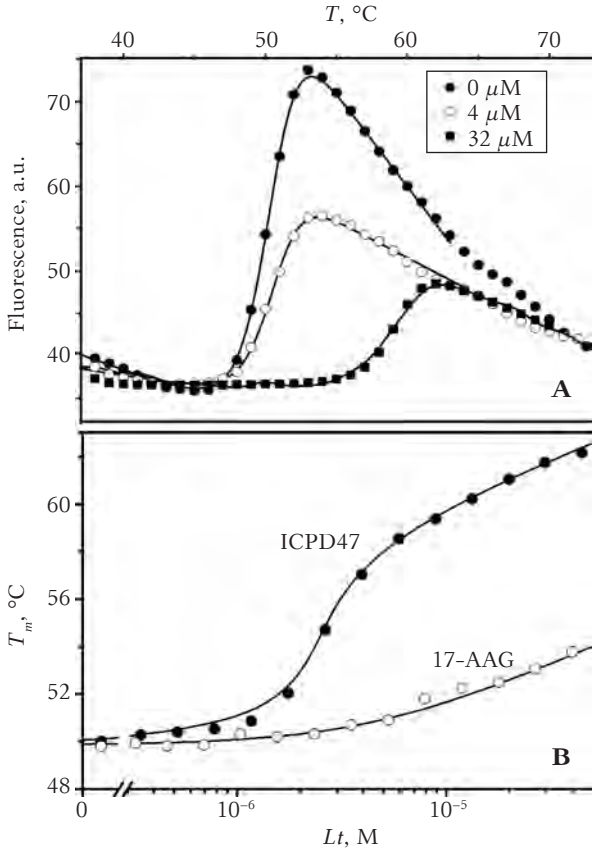


Fig. 5. Inhibitor binding to Hsp90 by TSA

Panel A, fluorescence curves of ICPD47 binding to Hsp90αN at pH 7.5. The Hsp90αN thermal denaturation transitions (T_m) were increasingly shifted upward as the concentration of ICPD47 increased. Panel B, dependencies of Hsp90αN melting temperature on 17-AAG and ICPD47 concentrations. The observed K_ds were 0.3 μM for 17-AAG and 0.002 μM for ICPD47.

The Cl atom between the OH groups interferes with binding.

In Vitro Anticancer Activity of Hsp90 Inhibitors

After the determination of intrinsic thermodynamic parameters of binding and x-ray crystallographic structures of the most potent inhibitors, it is necessary to test the compounds against several cancer cell lines. The effect of ICPD compound 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) – using tetrazolium/formazan assay (Fig. 6) (57). ICPD60 was relatively weak and exhibited off-target activity against the cancer cell lines. This property correlates well with its weak binding to Hsp90. Other compounds exhibited the average potency of cancer cell growth inhibition. The compound series has potential to become candidates for therapeutic anticancer treatment.

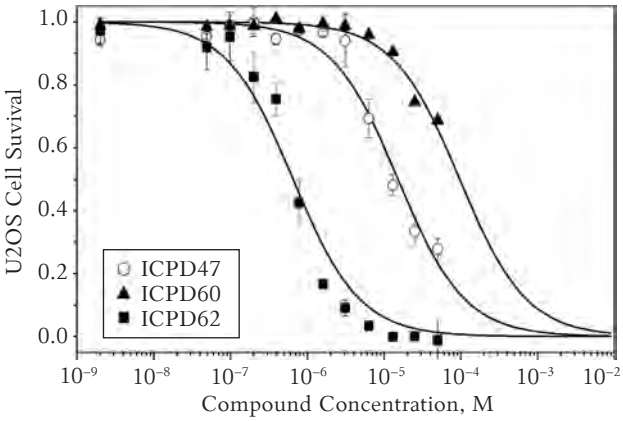


Fig. 6. Normalized U2OS cancer cell line survival as a function of compound (ICPD47, ICPD60, and ICPD62) concentration

Concluding Remarks

Hsp90 is a prominent anticancer target. The ICPD series of compounds are highly potent inhibitors of Hsp90 (single digit nanomolar K_d). Biothermodynamic methods – isothermal titration calorimetry and thermal shift assay – are useful in the

characterization of compound binding efficiency to the target.

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Statement of Conflict of Interest

The authors state no conflict of interest.

Natūralių ir sintetinių slopiklių jungimasis su žmogaus baltymu Hsp90 ir jų pritaikymas klinikoje

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Raktažodžiai: Hsp90 slopikliai, priešvėžinis aktyvumas, izoterminio titravimo kalorimetrija, šiluminio poslinkio metodas, ThermoFluor®.

Santrauka. Straipsnyje pateikiama Hsp90 slopiklių kūrimo apžvalga. Hsp90 yra šiluminio šoko baltymas, kurio santykinis molekulinis svoris yra apie 90 kDa. Hsp90 yra laikomas tinkamu priešvėžinių vaistų taikiniu, nes, jį užslopinus, daugelis klientinių baltymų, dalyvaujančių įvairiuose signalo perdavimo ir kituose vėžio vystymosi procesuose, tampa neaktyvūs. Šioje apžvalgoje aprašyti Hsp90 slopikliai, šiuo metu tiriami klinikiniuose tyrimuose.

Taip pat apžvelgiami biofizikiniai metodai, naudojami kuriant Hsp90 slopiklius. Sukurtų junginių lyderių jungimasis su įvairiais Hsp90 konstruktais gali būti matuojamas izoterminio titravimo kalorimetrijos ir šiluminio poslinkio metodais. Taikant šiuos metodus, galima ištirti jungimosi reakcijų energetiką ir nustatyti tokius biofizikinius parametrus, kaip entalpiją, entropiją, šiluminę talpą, laisvąją Gibso energiją. Išsamios žinios apie jungimosi energetiką suteikia daugiau informacijos apie struktūros ir aktyvumo ryšį, aktyvesnių slopiklių kūrimą. Vėliau tiriamos aktyviausių junginių ADME-TOX savybės ir jų gebėjimas slopinti vėžinių ląstelių augimą.

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