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Kinetics of Chaperone Activity of Proteins Hsp70 and Hdj1 in Human Leukemia U-937 Cells after Preconditioning with Thermal Shock or Compound U-133

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Abstract—Kinetics of the chaperone activity of proteins Hsp70 and Hdj1 were analyzed in human U-937 promonocytes during their response to heat shock or to treatment with the echinochrome triacetyl glucoside derivative U-133. To measure the chaperone activity of both proteins, a special test was developed for their recognition and binding of a denatured protein. Using this test, the chaperone activity could be concurrently estimated in large numbers of cellular or tissue extracts. We also estimated the contents of both chaperones in cells by immunoblotting. The values for contents of Hsp70 and Hdj1 obtained by two independent test systems coincided, and this suggested that the substrate-binding activity could change proportionally to the chaperone content in the protein mixture. Therefore, the test developed by us can be employed for high throughput screening of drugs activating cellular chaperones. The analysis of quantity and activity of two cellular chaperones during the cell response to heat stress or to the drug-like substance U-133 showed that both factors caused the accumulation of chaperones with similar kinetics. We conclude that the efficiency of drug preconditioning could be close to the efficiency of hyperthermia and that the high activity of chaperones could be retained in human cells for no less than 1.5 days.

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Physicians now pay increasing attention to molecular chaperones because approaches based on them can be used for suppression of the development of many pathological processes in the organism. Just the chaperone activity controls mechanisms of assembly of protein structures and prevents aggregation of damaged or mutant proteins. These aggregations cause such severe diseases as Alzheimer's disease, Huntington's chorea, amyotrophic lateral sclerosis, etc. Development of approaches based on our knowledge about chaperones has already produced notable results. Thus, the chaperon Hsp90 has become a target for antitumor preparations, such as geldanamycin and its derivatives; some of them are at the third stage of clinical testing [1]. Note that these substances induce synthesis of another important cellular chaperone, Hsp70, which promotes the protection of cells against various cytotoxic and stress factors. The cytoprotective activity of Hsp70 and of some chaperones of the J-domain-containing proteins, Hdj1-3 and some others, is convincingly shown in many works on cellular and animal models of different diseases.

Naturally, these data stimulate the search for new approaches to increase chaperone function of cells and/or the content of chaperones themselves. An approach for increasing chaperone expression has been known for a long time. It is moderate hyperthermia, or preconditioning. Recently clinicians also began to use electromagnetic fields and laser radiation with particular wavelengths or pulse parameters [2-4]. Chaperone-inducing activity is observed in the above-mentioned gel-

Abbreviations: CMLA, carboxymethylated lactalbumin; DTT, dithiothreitol; EIA, enzyme immunoassay; PBS, phosphate-buffered saline; U-103, tetra-O-acetyl-β-D-glucopyranoside; U-133, echinochrome triacetyl glucoside.

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danamycin compounds, a preparation of the Far Eastern medicine celastrol, a synthetic membranotropic preparation arimoclomol, etc. [5, 6]. We have added recently to this list of chaperone inducers derivatives of echinochrome triacetyl glucoside (U-133) [7] and of shikonin acetyl glucoside (U-103) [8], which increase the content of Hsp70 in human erythroleukemia K-562 cells and display a pronounced cytoprotective effect [9].

The protective effect of heat shock proteins is mainly based on their chaperone activity; therefore, it is necessary to understand how it changes in the cells treated with chaperone inducers and during what time it is sufficiently effective in protecting the cells. The preparation U-133 was shown to be less cytotoxic than U-103 [9], and so it was chosen for the present study. We observed earlier an unusually prolonged, up to 3 days, existence of Hsp70 in cells after a "conditioning" heat shock [10]. It was supposed that over this time the chaperone should exist in a complex with other proteins, and that this complex, although dynamic, should be unusually resistant to cellular proteolytic enzymes [11]. It seems very important that the retention of the high level of Hsp70 is associated with resistance of the cells to various cytotoxic factors, possibly, due to binding to chaperones of proapoptotic proteins [12].

The purpose of this work was to analyze the state of the chaperone system Hsp70-Hdj1/Hsp40 during the reaction of the human U-937 promonocytes to heat shock as a classic inducer of the chaperone system and of the chosen compound U-133.

MATERIALS AND METHODS

Cells. Human myeloid leukemia U-937 cells were obtained from the Russian Collection of Cell Cultures (Institute of Cytology, Russian Academy of Sciences, St. Petersburg) and were maintained in RPMI-1640 medium supplemented with 10% calf embryonic serum (Paneko, Russia) at 37°C in the presence of 5% of CO_2. On the day before the experiment the cells were transferred into fresh medium; the initial concentration was $0.2 \cdot 10^6$ cells/ml. For experiments cultures were used with fraction of dead cells not exceeding 5%. To induce accumulation of Hsp70, the cells were subjected to heat shock at 43°C for 60 min. The preparation of U-133 (0.5 μM) was injected into the culture. After some time the cells were subjected to lysis in RIPA buffer solution (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100), the protein concentration was determined in the extracts [13], and 30 μg protein from each specimen was used for preparing samples for electrophoresis, and the remainder was used for determination of the chaperone activity of Hsp70 and Hdj1 with a special test system described below.

Proteins and antibodies. Hsp70 and Hdj1 were isolated from bacteria transformed, respectively, with plasmids pMSHsp70 and pGEX4T1 Hdj1 (both were presented by Prof. R. Morimoto, Evanston, USA). Hsp70 was purified using two-step chromatography according to the previously developed protocol [14]. The Hdj1 protein was purified from the bacterial extract using affinity chromatography on glutathione-Sepharose gel; after loading the bacterial protein and column washing as described in [15], the protein was separated from the gel by incubation with 2.5 μM thrombin at pH 6.0.

Polyacrylamide gel electrophoresis revealed that the purification of both proteins was no less than 95%.

Immunoblotting. The proteins were separated by SDS-disc-electrophoresis in 10% polyacrylamide gel, and bands of the separated proteins were transferred onto a Whatman nitrocellulose membrane (Sigma-Aldrich, Germany) with a Trans-Blot apparatus (BioRad, Russia) according to standard protocols. The Hsp70 and Hdj1 bands were detected in the blots using specific monoclonal antibodies 3B5 and J32, respectively, prepared in the laboratory, and secondary antibodies conjugated with horseradish peroxidase. The peroxidase reaction was visualized by enhanced chemiluminescence.

Determination of substrate-binding (chaperone) activity. The chaperone activity of Hsp70 was measured using our modification [11] of enzyme immunoassay described in work [16]. The substrate target protein carboxymethylated lactalbumin (CMLA) was prepared by denaturation of lactalbumin by incubation in 8 M urea, and then the protein solution was supplemented with dithiothreitol (DTT). Then the alkylation reaction was triggered by introduction into the protein solution of iodoacetamide and stopped after 24 h by addition of DTT. The resulting CMLA was immobilized in the wells of a 96-well plate at the concentration of 10 μg/ml in phosphate-buffered saline (PBS). To inhibit nonspecific binding, into the wells of the plate 0.3% solution of BSA (Sigma, USA) in PBS and then the cellular extracts in the concentrations of 10 and 30 μg/ml were introduced. Hsp70 or Hdj1, which are components of the protein mixture of the extracts, bound with the denatured target protein. Then a solution of affinity-purified polyclonal RAF-6- or monoclonal J32-antibodies, respectively, was introduced into the wells. The peroxidase activity was determined as described earlier [9].

RESULTS

To determine changes in the amounts of active cellular chaperones Hsp70 and Hdj1 during the cell response to conditioning by the heat shock and or by treatment with the promising drug U-133, two approaches were used: EIA with a test system capable of detecting substrate-binding chaperones and immunoblotting for assessment of the total protein amount in the extracts. We modified the chaperone system CHA-EIA described in

work [16] for analyzing properties of pure chaperone DnaK for measurement of the content of pure Hsp70 in the samples of extracts prepared from cells and tissues [9, 11]. In particular, the purpose of this study was to show advantages of the new test system for analysis of the chaperone activity of two proteins, Hsp70 and Hdj1. The test is based on the recognition by the chaperone of a substrate represented by a denatured protein, in this case CMLA. We found earlier that the substrate-binding activity of Hsp70 could be determined with this test in cellular extracts, i.e. in complex protein mixtures [11]. There was no such test system for Hdj1, and because this protein seemed to be involved in the primary recognition of a substrate polypeptide before it passing to Hsp70, we suggested that this test could be also used for measurement of the co-chaperone concentration. To test this hypothesis, we used an extract of bovine muscle, which according to data of immunoblotting contained significant amounts of both chaperones (unpublished data). The extract was added into parallel rows of wells with the immobilized CMLA on the bottom, and then a solution of 0.3% albumin was introduced to inhibit nonspecific binding. Upon incubation of the extract, polyclonal antibodies to Hsp70 and the antibodies J32 recognizing Hdj1 were introduced, respectively, into one and the other row of wells. The efficiency of the substrate binding was assessed by intensity of the reaction of peroxidase conjugated with secondary antibodies to rabbit and mouse immunoglobulins. The measurements revealed that Hdj1 had a substrate-binding activity detectable in the extract on its dilution to the total protein concentration of 100 μg/ml (table). Note that on an increase or decrease in the concentration of total protein in the extract both parameters changed: we think that this confirmed the competence of the test.

After the new test system was shown to function correctly, we determined the limits of its sensitivity for both chaperones. Hsp70 and Hdj1 were prepared from biomass of bacteria transformed by genes of the corresponding human proteins. The purified proteins were introduced in the wells of a plate with the immobilized substrate, CMLA, and the amount of the bound material was determined using antibodies to Hsp70 and Hdj1. Data of CHA-EIA indicated that both chaperones effectively bound with the substrate protein, and the lower concentration for producing a signal was 5-10 ng/ml. Steady high values of the activity were observed at the chaperon concentration >25 ng/ml, and both values flatten out at concentration >100 ng/ml (Fig. 1).

Verification of competence of the test for substrate-binding activity in the case of Hsp70 and Hdj1 proteins

Protein concentration in muscle extract, μg/ml	Absorption at 450 nm	
	anti-Hsp70	anti-Hdj1
0 (without extract)	0.01 ± 0.01	0.01 ± 0.01
5	0.22 ± 0.04	0.05 ± 0.01
20	0.77 ± 0.06	0.11 ± 0.02
100	1.94 ± 0.14	0.41 ± 0.06

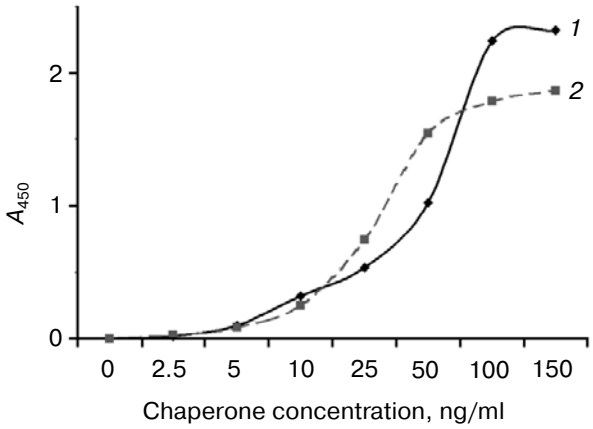

Fig. 1. Determination of threshold sensitivity of the test system CHA-EIA. *1*, *2*) Preparations of purified Hsp70 and Hdj1, respectively. Peroxidase activity (A_{450}) was determined according to the standard protocol (see "Materials and Methods").

On analyzing changes in the chaperone amounts in the cells after the preconditioning, we used immunoblotting, which allowed us to measure the total amount of protein in the cell and the test CHA-EIA. For these studies the U-937 cells were subjected to heat shock or incubated with the preparation U-133, and then samples of the cells were taken for both types of analysis. Fractions of the extracts were used for preparing specimens for electrophoresis, which were placed in equal quantities on the gel paths, and after the protein bands were transferred onto the membrane, they were stained using antibodies 3B5 and J32 to Hsp70 and Hdj1, respectively. Typical immunoblots are presented in Fig. 2. It is obvious that both heat shock and the substance U-133 are effective inducers of Hsp70 and Hdj1, and the contents of both proteins remain high during at least 24 h (Fig. 2). The content of Hsp70 in the heat-treated cells increased approximately 3 h earlier and the protein itself remained in the cells for a longer time than after the treatment with U-133. The content of Hdj1 changed similarly, but this parameter was maintained at an increased level for a longer time after the treatment with U-133 (Fig. 2).

Similar results were obtained on the assessment of the substrate-binding activity of the proteins. Note that an increase in the amount of the active chaperone Hsp70 started 1 h after the heat shock and return to the initial values occurred within 24 h; on the treatment with U-133 the kinetic curve of the process was shifted to the right (Fig. 3). The same differences were characteristic for

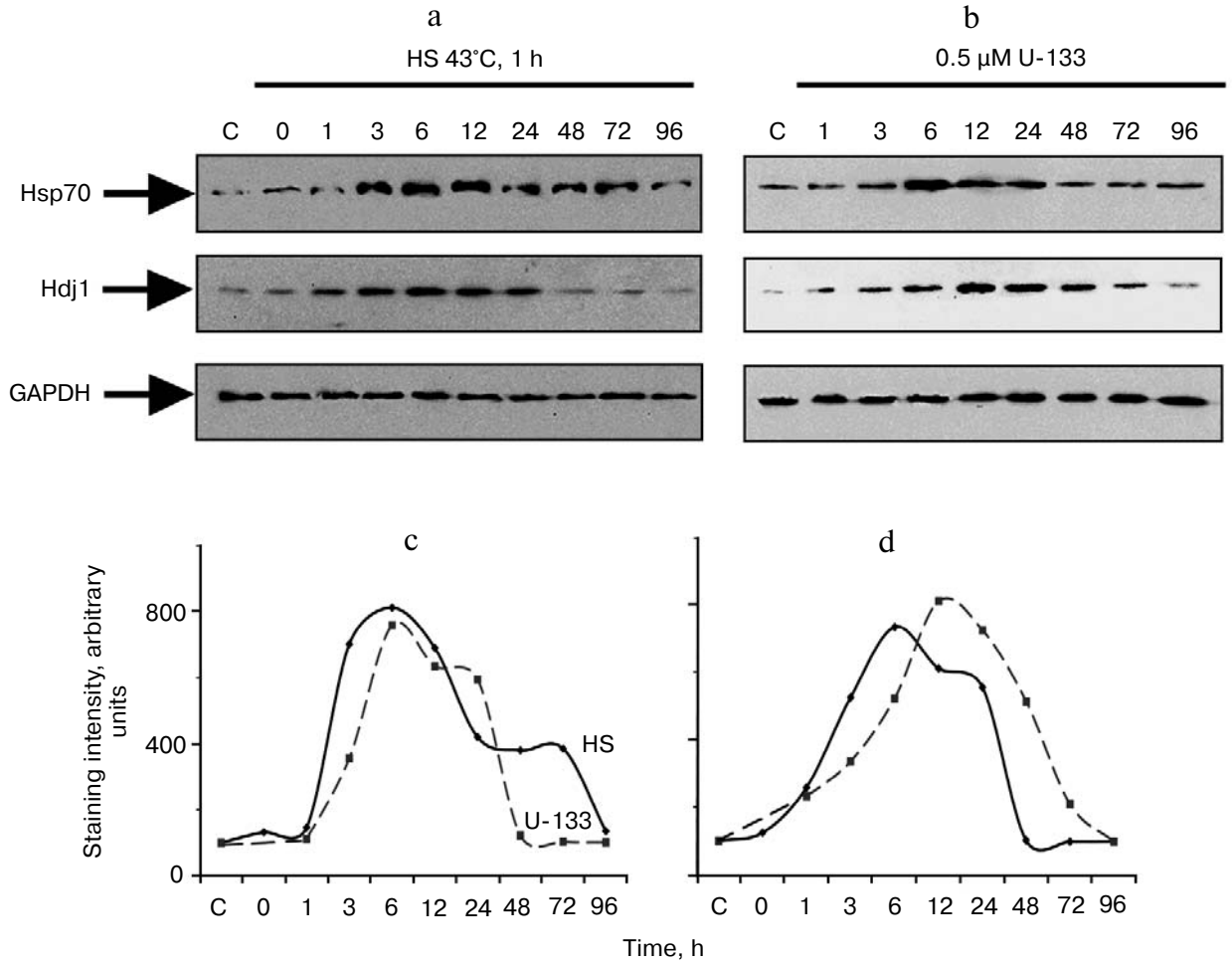

Fig. 2. Changes in amounts of chaperones Hsp70 and Hdj1 in human leukemia cells U-937 after heat shock (c) and after treatment with U-133 (d). The cells were heated to 43°C for 1 h (a) or incubated with U-133 (0.5 μM) (b), then at the indicated time intervals they were washed and extracted to prepare samples for electrophoresis in SDS-polyacrylamide gel. The protein bands transferred onto nitrocellulose were stained with antibodies 3B5 and J32 capable of recognizing, respectively, proteins Hsp70 and Hdj1. In the upper and lower parts of the figure stained blots and scanning results are presented, respectively. C, control; HS, heat shock.

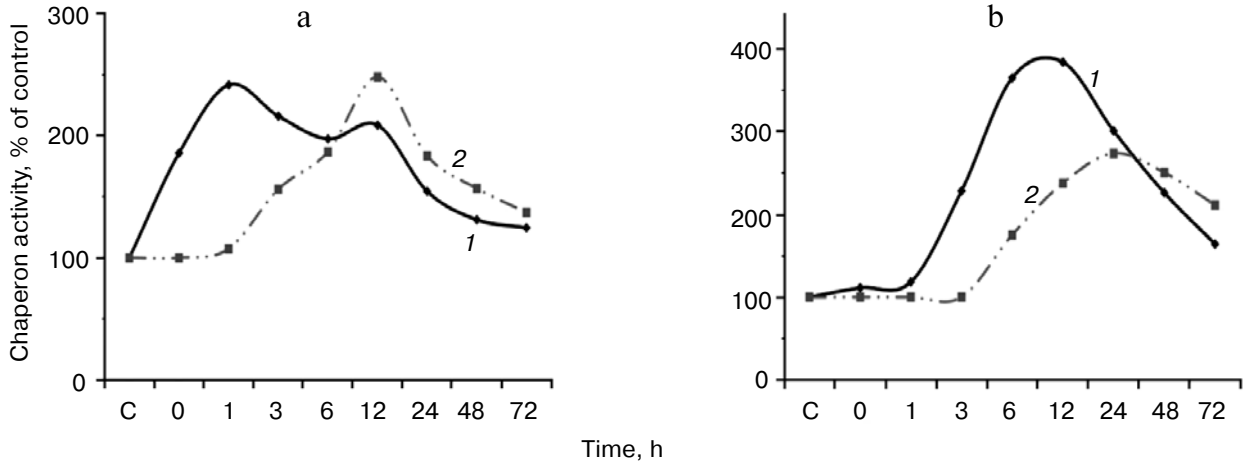

Fig. 3. Profiles of substrate binding activity of chaperones Hsp70 (*1*) and Hdj1 (*2*) in human promonocytes U-937 after heat shock (a) and after treatment with U-133 (b). Samples of the cells were prepared and the chaperone activity with the CHA-EIA test was analyzed as in the experiments shown in Figs. 1 and 2. The chaperone activity of the preparations was determined as the enzymatic activity of peroxidase in optical density units at 450 nm and presented as percent of control for untreated cells taken as 100%.

changes in the amount of active Hdj1: the kinetic curve was shifted to the right, suggesting a delay of this process in the cells treated with U-133. On comparing the approaches for characterizing the chaperones, it should be noticed that the kinetic curves of amounts and substrate binding activity of Hsp70 and Hdj1 after the heat shock were slightly different. Thus, the decrease in the chaperone activity of Hsp70 was faster than the decrease in the protein content in the cells (Figs. 2 and 3).

DISCUSSION

Synthesis of Hsp70 and Hdj1 proteins is triggered by activation of the heat shock factor HSF-1 in response to an influence capable of damaging the structure of cellular polypeptides [17]. It is important that the systems based on these two chaperones play in the cell a protective role preventing the aggregation of stress-damaged polypeptides and supporting protein homeostasis in the cell [18]. Obviously, the higher the chaperone potential of the cell, the higher are its chances to survive in the struggle with a huge number of endo- and exogenous cytotoxic factors including the so-called protein pathogens: β-amyloid (Alzheimer's disease), α-synuclein (Parkinson's disease), huntingtin (Huntington's chorea), etc. Therefore, compounds inducing accumulation of the above-mentioned proteins in cells and tissues are now considered as possible therapeutic preparations.

It has been recently shown that acetyl glucoside derivatives of shikonin and echinochrome, U-103 and U-133, not only induce synthesis of Hsp70, but also increase the cell resistance to damaging effect of severe heat shock, free radicals, and staurosporine [9]. To provide for long-term resistance of cells, the chaperone activity must be maintained at a high level also for a long time, and we have tested if this requirement is realized for the preparation U-133 as compared to heat shock, which is a traditional inducer of stress proteins. We used the CHA-EIA test-system, which we modified earlier for determination of Hsp70 concentration in complex protein mixtures [11] and of the concentration of Hdj1 in the present work. As expected, the CHA-EIA test can be also used for Hdj1.

Thus, our data confirm the appropriateness of a chaperone mechanism model where Hdj1 binds with a substrate protein earlier than Hsp70. Possibly, this binding is necessary to allow a substrate to come to the major chaperone in a certain conformation [19]. Note that for the correct functioning of this mechanism, the protein Hdj1 has to recognize the EEVD motif on the C-terminal part of the Hsp70 molecule, and the presence of ATP is necessary [15]. Note that both works were performed using purified recombinant proteins. We found earlier that addition of ATP into the incubation medium (an extract of the K-562 cells) placed into the wells containing immobilized CMLA resulted in a nearly complete disappearance of the signal in the case using antibodies against Hsp70 [11]. According to the preliminary data, this did not occur when in this test antibodies against Hdj1 were used. Consequently, using the *ex vivo* system we confirmed that interaction mechanisms of the two chaperones with the substrate are essentially different [19].

The data presented in the table and in Fig. 1 suggest that chaperone concentrations at which the binding with substrates becomes noticeable, i.e. 5-10 ng/ml, are closely corresponding to the sensitivity of the test systems proposed by the Canadian company StressMarq (www.stressmarq.com) for measurements of contents of the Hsp90 and Hsc70 chaperones in biological fluids. These tests are based on essentially different principles. The systems produced by the Canadian Company seem to be based on the sandwich principle, i.e. antibodies which capture an antigen are immobilized in the wells of the plate, whereas the upper antibodies recognize the other part of the antigen, i.e. there are capture and detection antibodies, respectively. And in such a system virtually all antigen molecules are detected. This approach is quite comparable with immunoblotting used for specific analysis of molecules with molecular weight of 70 and 40 kDa and carrying antigenic determinants, respectively, to Hsp70 and Hdj1 (Fig. 2). In our test chaperones recognize an irreversibly denatured protein, and then they in turn are detected by antibodies. Under these conditions the test reveals only active chaperones, which mainly react with an immobilized substrate protein. Note that both tests give close although not identical curves of changes in the amounts (Figs. 2 and 3). Such a discrepancy is especially characteristic for Hdj1, the activity of which is sufficiently high at the point of cell incubation for 72 h, and the data of immunoblotting show that at this point the protein amount is already low. We supposed earlier that the substrate-binding activity can be influenced by other factors; thus, for Hsp70 such a factor can be represented by the balance between Hdj1 and the protein Bag-1 realizing nucleotide substitution [11]. It seemed that this protein Bag-1 whose influence on the activity of the major "player" Hsp70 was stronger at further stages of K-562 cells to heat stress could also support the high activity of its antagonist Hdj1 by a feedback mechanism. At least, it does not contradict our ideas about the operation of the chaperone mechanism based on Hsp70-class proteins [20].

Considering the data on changes in the contents of the two proteins under the influence of heat shock as a traditional inducer and of the drug U-133, we conclude that at least for 24-36 h monocytic cells retain a sufficient amount of chaperones that determines their resistance to damaging action of various stress factors and pathogens (also see [9]). Thus, the proposed method of concurrent analysis of two chaperones, CHA-EIA, being adapted to work with complex protein mixtures, blood sera, or tissue extracts (biopsy) is promising for molecular pharmacology directed to creating small molecules with multiple functions.

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