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Establishment of a High-Throughput Screening System for Caspase-3 Inhibitors

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In most tissues, apoptosis plays a pivotal role in normal development and for regulating cell number, thus inappropriate apoptosis underlies a variety of diseases. Caspase-3 is one of a family of caspases that are mainly involved in the apoptotic signal transduction pathway, where caspase-3 acts as an effect molecule to proteolytically cleave intracellular substrates that are necessary for maintaining cell survival. Recent evidences show that apoptotic cell death can be blocked by inhibiting caspase-3, suggesting its inhibitors have potential to be therapeutic drugs for the diseases related with inappropriate apoptosis. We have established a screening system to search caspase-3 inhibitors from chemical libraries stocked in our institute. The enzyme assay is configured entirely in 96-well format, which is easily adapted for high throughput screening. Before performing mass screening, 80 in-house compounds were screened as a preliminary experiment, and we found that morin hydrate inhibited caspase-3 by 66.4% at the final concentration of 20 μM.

Key words: Apoptosis, Caspase-3, High throughput screening

INTRODUCTION

Apoptosis, or programmed cell death, is a physiological process important in normal development and for maintaining homeostasis of an organism by removing cells that are damaged or have become inordinately excessive (Jacobson *et al.*, 1997). Abnormal apoptosis has been implicated in the pathogenesis of various diseases, which are occurred from inappropriate cell death. Parkinsons disease, stroke, Alzheimers disease are derived from uncontrollable death of neuronal cells (Kostic *et al.*, 1997, Anglade *et al.*, 1997; Cotman *et al.*, 1995; Hara *et al.*, 1997; Johnson *et al.*, 1995). The main reason of immunodeficiency in AIDS patients is reduced number of $CD4^+$ helper T cells due to its severe cell death (Groux *et al.*, 1992; Pandolfi *et al.*, 1993). On the contrary, tumors are developed from lack in death of cells which should be destined to die (Thompson, 1995). In this regard, molecules capable of intervening the apoptosis pathway can become potent therapeutic drugs for above diseases.

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Apoptosis is characterized by controlled proteolysis of cellular components resulting from activation of death signal. While the sources of death signals are as diverse as ionizing radiation, chemotherapeutic drugs, block of supply of growth factors, and ligation of specific death receptor, the key features of intracellular process appear to be quite similar: the death signal induces the activation of a number of proteases, which in turn cleave protein substrates in a cascade manner (Nagata, 1997).

Caspases are a family of cysteine protease, which preferentially cleave substrates after aspartic acid while having cysteine residue in its catalytic site and play important roles in apoptotic pathway. Caspases are categorized as three groups based upon their function and sequence homology. Caspase-1, 4, and 5 are involved in group I, the role of which is to turn on the signals for apoptosis as well as to produce the interleukin-1β. In group II, which are involved in transmitting signals for activation of other caspases, are caspase-2, 8, 9, and 10. The final execution of cell death by cleaving substrates such as poly (ADP-ribose) polymerase (PARP), lamin A, and actin which are required for maintaining cell integrity and survival are performed by caspase-3, 6, and 9, which are classified into group III (Alnemri *et al.*, 1996).

Caspase-3 (CPP32/Yama/apopain), like other caspase

family members, consists of two large and two small subunits, and recognizes specific sequences on its substrates such as PARP, rho-GDI, SREBP, and DNA-dependent protein kinase (Takahashi *et al.*, 1996, Nicholoson *et al.*, 1995). The fact that acetyl-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-fmk), a peptide competing for specific recognition sites on substrates for caspase-3, along with its caspase-3 inhibitory activity, blocks cell death (Fearnhead *et al.*, 1995) strongly suggest that caspase-3 is an ideal target to block cell death process. Another interesting feature of caspase-3 is that it is positioned downstream in the cascade of caspases-mediated signal transduction pathway, which let us guess that molecules capable of inhibiting caspase-3 may have potential as therapeutic agents with relatively less side effects and they barely affect other enzymes involved in other pathways. Recent studies from the mice deficient in caspase-3 also suggest its possible stringent effects on neuronal cells in vivo. The caspase-3 deficient mice die perinatally with a vast excess of cells in their central nervous system, apparently as a result of decreased apoptosis in neuronal cells, although apoptosis in other organs seems to occur normally (Kuida *et al.*, 1996).

Although specific competitive peptide inhibitors, and some proteins encoded by viral genes such as crmA (Ray *et al.*, 1992) or p35 (Bump *et al.*, 1995; Xue and Horvitz, 1995) have been shown to block cell death by inhibiting caspase enzymes, they have intrinsic defects for development as pharmaceutical drugs. Thus, the development of small molecules to inhibit specifically caspase-3 would be of value as potent therapeutic drugs to cure diseases derived from excessive cell death. Herein we report the establishment of high throughput screening system for caspase-3 inhibitors.

MATERIALS AND METHODS

Preparation of human recombinant caspase-3 enzyme

The active form of human recombinant caspase-3 enzyme was prepared from *Escherichia coli* (*E.coli*) heterologous expression system as described previously (Rotonda *et al.*, 1996). Briefly, each cDNA encoding p12 and p17 subunits of caspase-3 was PCR-amplified respectively and subcloned into a pET3-a expression vector (Novagen), which was followed by transfection into *E.coli* strain BL21 (DE3). The bacterial cultures (0.5 liters for each transfectants) were grown at 37°C until logarithmic phase of cell growth and isopropyl-l-thiol-β-D-galatopyranoside (IPTG) (Pharmacia) was then added to the final concentration at 0.5 mM. After 3 hr more incubation, the bacteria were pelleted and lysed in a lysis buffer (20 mM Tris · HCl pH 8.0, 1 mM EDTA, 2 mM dithiothreitol (DTT), 100 mM NaCl, 200 μg/ml of lysozyme) through sonication. After centrifugation, the pellets from each transfectants were dissolved in 6 M urea and were mixed and rapidly diluted to 100 mM HEPES/KOH (pH 7.5), 10% sucrose, 0.1% CHAPS, 0.5 M NaCl, 10 mM DTT by 50 fold. The concentration of protein was determined with the Bradford method (Bio-Rad), with bovine serum albumin as standard. To verify whether expected size of proteins were obtained, the cell lysates from each cDNA transfectants were analyzed on 14% SDS PAGE and visualized with Coomassie blue staining.

Substrates

The fluorogenic substrate benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin (Ac-DEVD-AFC) were purchased from Enzyme Systems Products and dissolved in dimethylsulfoxide (DMSO) as a 2.5 mM stock solution and kept at -20°C before using.

Enzyme assays

For reasons discussed later, the standard assays contained in 200 μl in each well of 96-well plate: 20 mM HEPES/KOH (pH 7.5), 10% sucrose, 10 mM DTT, 0.2 mM EDTA, 0.1% CHAPS, and 200 ng of enzymes and substrate with final concentration of 2.5 μM were added. After an 1 hr incubation at 37°C, the release of AFC was monitored in a fluorometric plate reader (Fluoroskan Ascent, Labsystems, Nedd ham Heights, MA). The excita-tion and emission wavelengths were 400 nm and 510 nm respectively. For kinetic analysis, the enzyme assays were performed with same conditions with various incubation time: the AFC release was monitored after 0.17, 0.5, 1, 2, 4, 5, and 24 hr incubation. To determine the optimal combinations of enzyme and substrate, checkerboard-like assays were done with various concentrations of enzyme and substrate. The amounts of enzyme tested were started from 50 ng with 2 fold increase whereas the final concentrations of substrate tested were 0.025 mM, 0.5 mM, and 1 mM.

The effects of organic solvents and pH on the enzyme activities

The effects of organic solvents such as DMSO, dimethylformamide (DMF), methanol, and ethanol on enzyme activities were determined by adding various degrees of concentrations of each solvent. The enzyme assays were performed as same conditions described above. The effects of samples with extreme pH values were tested by using hydrochloric acid (HCl) and sodium hydroxide (NaOH) solution.

Validation of enzyme assay for drug screening

To validate this enzyme assay for drug screening, 80 compounds collected from in-house stores were screened

before performing mass screening. The total of 80 compounds are listed at Table I. The compounds were dissolved in appropriate solvents at a final concentrations of 10 mM and dispensed into a 96 well-plate, which were designated as a stock plate. The screening was done by transferring 20 µl from stock plate to a new 96-well assay plate, and followed by addition of 80 µl of substrate solutions (substrate, 2 × assay buffer, and deionized water (D.W.)). After homogenizing the sample and substrate by plate mixing, 100 µl of enzyme solutions (200 ng of enzyme, 2 × assay buffer, and D.W.) were added and incubated for 1 h.

RESULTS AND DISCUSSION

Since our purpose in establishment of screening system with caspase-3 enzyme assay is to develop caspase-3

Table I. The list of compounds for screening for caspase-3 inhibitors

No.	Well[a]	Compounds	No.	Well	Compounds
1	3A	Cefotaxime	41	3E	Ceftriaxone
2	4A	Cephapirin	42	4E	Methyl-a-D-mannopyranoside
3	5A	Phloretin	43	5E	Busulfan
4	6A	Imidazole	44	6E	Lemakalim
5	7A	BMS-180448	45	7E	Tazobactam
6	8A	Ellipticin	46	8E	Epigallocatechin gallate
7	9A	Ftofur	47	9E	Trimethoprim
8	10A	Erythromycin	48	10E	Amphotericin B
9	11A	B-NAD	49	11E	Spermine tetrahydrochloride
10	12A	Indomethacin	50	12E	Cefuroxime
11	3B	Vancomycin	51	3F	Piperacillin
12	4B	Streptomycin sulfate	52	4F	Dexamethasone
13	5B	Rhapontin	53	5F	Epicathechin
14	6B	Papaverine	54	6F	Dup 753
15	7B	Amantadine	55	7F	Mitoxantrone
16	8B	Vinblastin	56	8F	Gallocatechin gallate
17	9B	Colchicine	57	9F	5-Fluorouracil
18	10B	DL-aminoglutethimide	58	10F	Bergenin
19	11B	Aspirin	59	11F	Glycerrhizic acid
20	12B	DL-a, B-diaminopimelic acid	60	12F	Thiamine monophosphate chloride
21	3C	Cefsulodin	61	3G	Ceftazidime
22	4C	Novobiocin	62	4G	Clinafloxacin
23	5C	Ipratropium bromide	63	5G	Captopril
24	6C	Hexamethodium Chloride	64	6G	Nalidixic acid
25	7C	Nifedipine	65	7G	Doxorubicin
26	8C	Myricetin	66	8G	Actinomycin D-mannitol
27	9C	Rotenone	67	9G	Podophyllotoxin
28	10C	Verapamil	68	10G	Nobergenin
29	11C	Ectoposide	69	11G	Rifampicin
30	12C	Penicillin G	70	12G	Tetracyclin
31	3D	Cefoxitin	71	3H	Cefotiam
32	4D	Hexaminocobalt (III) chloride	72	4H	Cloxacillin
33	5D	Taurin	73	5H	Methoxamine
34	6D	Dilitazem	74	6H	Cephaloridine
35	7D	a-cyclodextrin	75	7H	Paclitaxel
36	8D	Epigallocatechin	76	8H	N-acetyl-cysteine
37	9D	Cytosine b-D-arabino-furanoside	77	9H	Vincamine
38	10D	Morin hydrate	78	10H	Spermidine
39	11D	8-HO-quinoline	79	11H	Tetrandrine
40	12D	Cephalothin	80	12H	Cefoperazone

[a] 'Well' indicates each position of compounds in 96-well plate

inhibitors from large chemical libraries, economical gain of target enzymes is one of prerequisite factors. To this end, caspase-3 enzymes were obtained with *E.coli* heterologous expression system. The transcripts for caspase-3 are produced as a zymogen that should be cleaved to be active, and active form of caspase-3 is composed of p12 and p17. Each cDNA of p12 and p17 which was amplified with RT-PCR was cloned into pET-3a expression vector. The induction of target proteins was performed by addition of IPTG, and proteins accumulated as inclusion bodies were obtained by sonication under the lysis buffer. From the SDS-PAGE analysis with Coomassie blue staining, it was found that the induced protein of expected size was comprised more than 90% of total protein based on the band-intensity (Fig. 1A). Because induced proteins comprised in almost all total proteins, we assumed that another step such as column preparation to obtain purer enzymes as described (Mittle *et al.*, 1997) was unnecessary, and indeed we could obtain active caspase-3 enzymes by mixing each protein in 50 fold volume of refolding buffer. The activities as well as kinetics of caspase-3 were analyzed in the capacity to cleave the caspase-3

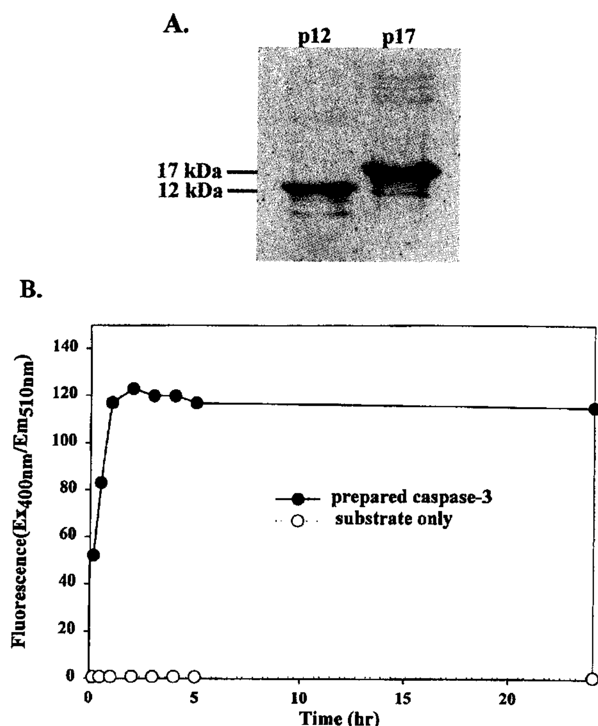

Fig. 1. Preparation of active caspase-3 enzymes with DNA recombination technology. A. The proteins of caspase-3 subunits (p12 or p17) which were expressed in pET-3a expression vector were prepared and analyzed onto 14% SDS-PAGE and visualized with Coomassie blue staining. The numbers on the left side indicate molecular weight of each protein. B. The prepared caspase-3 enzyme and its specific fluorogenic substrate were incubated at 37°C for various times. The enzymatic activity was expressed as the fluorescence emitted from free AFC liberated from substrate

specific fluorogenic substrate. The prepared caspase-3 could cleave all of substrate within 1 hr and even extending the incubation to 24 hr did not change much, whereas the fluorescence from substrates only in the absence of enzyme was not detected (Fig. 1B). To optimize assay conditions, a checkerboard-like assay was performed with various concentrations of enzyme and substrate. From the result of Table II, the combination of the 200 ng of total protein of caspase-3 and 2.5 μM of substrate as a final concentration was selected for further drug screening. With this combination the fluorescence signal was ranged from 100-130, which is somewhat intermediate level, but there is no difficulty in determination of 50% inhibition concentration (IC_{50}). And more importantly, with this combination, the amount of substrate which is one of major limiting-factor in mass drug screening in terms of running cost could be kept as minimal. As we have obtained about 20 mg of total proteins from the preparation of 500 ml of cultures of each transfectant, in case we use 200 ng for screening 1 compound, it is calculated that about 100,000 compounds can be analyzed with single preparation.

Since we are going to screen the versatile chemical libraries with previously unknown chemical properties, it is also important to test whether this enzyme assay is vulnerable to organic solvents and various pH ranges, which have potential to lead to false positive as well as negative results. All organic solvents, DMSO, DMF, methanol, and ethanol, used in this study, in less than 10% total volume barely affected the result of enzyme assay (Fig. 2A). The effect of HCl and NaOH in assay mixtures with various final pH values ranged from 1 to 12 was evaluated. When the 1N HCl and NaOH was mixed with assay mixtures by 10%, the resulting pH was 1.1 and 12.1 respectively, and enzyme activity was severely reduced by more than 90%. When the final concentration of HCl

Table II. A checkerboard-like assay for the determination of optimal concentrations of enzyme and substrate

Caspase-3 (ng)	Substrate (mM, final concentration)		
	1	0.5	0.025
0	0.4[a]	0.3	0.4
50	289.2	193.1	78.2
100	356.3	212.3	110.2
200	429.8	254.3	130.5
400	430.8	269.2	150.2
800	450.5	271.2	149.8

Different concentrations of prepared caspase-3 enzyme were incubated with different concentrations of its specific fluorogenic substrate, Ac-DEVD-AFC for 1 h at 37°C. The enzyme activity was expressed as the fluorescence emitted from free AFC liberated from substrate. The fluorescence was detected with a fluorometer with excitation and emission wavelength of 400 nm and 510 nm respectively.
[a]fluorescence (Ex_{400nm}/Em_{510nm})

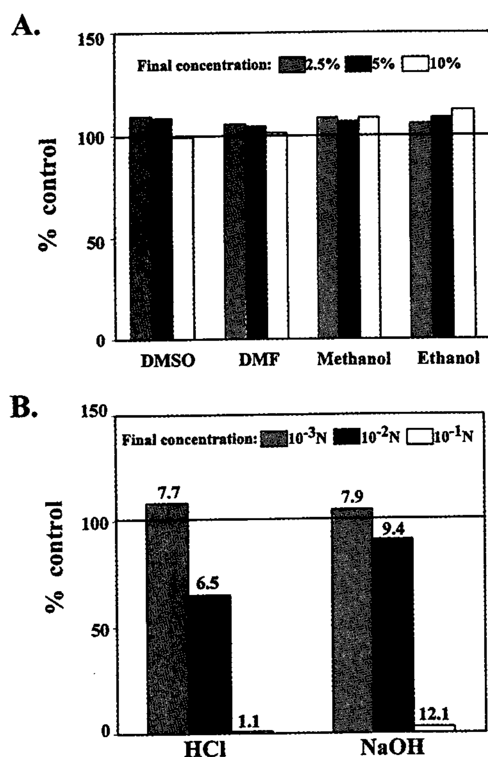

Fig. 2. The effect of chemical natures of compounds to enzyme activity. The prepared caspase-3 enzyme and its specific fluorogenic substrate were incubated at 37°C for 1 hr with various concentrations of four major organic solvents (A) or HCl, or NaOH (B). The enzymatic activity was expressed as the fluoescence emitted from free AFC liberated from substrate. In (B), the number on each bar indicates the final pH values of assay mixtures. Data represent % inhibition of control

was 0.01 N, the resulting pH was 6.5 and the enzyme activity was reduced by 60%, whereas pH 9.4 with 0.01 N NaOH did not affect results. The 0.001 N final concentration of HCl or NaOH barely affected the enzyme activity (Fig. 2B). Because samples to be assayed are usually contained at 10% total volume, except in the case that the samples have extreme pH values above 13 or less than 2, there should be no false results due to the samples themselves. Collectively, this enzyme assay is tolerant to various organic solvents and samples with various pH values. However, because samples with extreme pH values inhibit caspase-3 activity, hit molecules obtained from primary screening should be verified their chemical properties before more extensive studies are progressed.

Before applying our enzyme assay for mass screening, randomly collected 80 compounds were subjected for screening. These compounds are in-house stocks from various research laboratories in our institute. Anticancer drugs, antibiotics, and drugs for cardiovascular diseases are included in these compounds. The enzyme assay was performed in a single determination of final concentration at 20 μM with 2 step preparations. One step was to add substrate mixtures onto 96- well plate containing sample compounds. The next step was to add enzyme mixtures onto it, followed by incubation at 37°C for 1 hr. As shown in Table III, morin hydrate, which was located in 10D well of assay plate, inhibited caspase-3 by 66.7%, while the other rest compounds exhibited no significant inhibitory effect. It is noteworthy that morin hydrate, a flavonoid from Brazil wood, has been shown to be cytoprotective in several types of cells (Zeng *et al.*, 1998). When morin hydrate was added to cultured rat glomerular mesangial cells which were attacked by oxyradicals, protective effect was shown (Zeng *et al.*, 1994). Whether caspase-3-related apoptotic process is involved in the cytotoxicity by oxyradicals in mesangial cells is another interesting issue to be studied further. Although morin hydrate was shown to inhibit caspase-3, its effective concentration of 20 μM is relatively high, therefore the possibility to be developed as a therapeutic drug is extremely low. But it should be a valuable work to make more active compounds from derivatives of morin hydrate through a structure-activity relationship study.

Summary, we have established a high throughput screening system for caspase-3 inhibitors. This screening can be done on 96-well plate and easily applicable to automation due to the lack of washing and separation steps.

Table III. Results of screening for caspase-3 inhibitors

Row	Column Numbers									
	3	4	5	6	7	8	9	10	11	12
A	3.4	0.9	1.7	0.0	1.7	2.6	1.7	0.0	2.6	2.6
B	2.6	-2.6	0.0	0.9	0.0	-3.4	1.7	0.0	1.7	0.9
C	2.6	0.9	0.0	0.0	0.9	4.3	6.8	-1.7	2.6	0.9
D	2.6	-6.8	0.9	-1.7	0.0	2.6	0.9	66.7	2.6	3.4
E	2.6	0.9	4.3	0.9	0.9	1.7	1.7	17.1	-9.4	1.7
F	1.7	0.9	3.4	0.9	-0.9	2.6	3.4	1.7	0.9	2.6
G	0.9	0.9	0.0	0.9	7.7	0.0	1.7	0.9	15.4	6.0
H	1.7	1.7	0.0	1.7	21.4	1.7	-0.9	-11.1	-3.4	1.7

The 80 in-house samples, the lists of which are in Table II, at the final concentration of 20 μM were screened for caspase-3 inhibitors. Data represent % inhibition of control.

With this screening system, about 25,000 compounds stocked in chemical libraries in our institute are going to be screened in following work.

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