Development of cell death-based method for the selectivity screening of caspase-1 inhibitors

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Abstract Caspase-1 selective inhibitors are novel therapeutic agents for inflammatory diseases. Selectivity assays for caspases can be initiated with purified enzyme, making these assays very costly and time consuming. Therefore, there is a need to develop a fast and reliable cell-based assay, which can be used for the selectivity screening of multiple caspases in a biologically relevant context in a single assay. In this study, we have developed an assay in which DNA fragmentation, a hallmark of apoptosis, of Jurkat cell line was examined post induction with etoposide in the presence or absence of inhibitors of caspases 1, 3, 8, 9 and pan-caspase inhibitors. We observed that caspases-3, -8, -9 and pan caspase inhibitors resulted in significant inhibition of etoposide-induced DNA fragmentation. However, caspase-1 specific inhibitor failed to prevent DNA fragmentation, suggesting that either caspases belonging to caspase-1 family (1, 4 and 5) are not present in the Jurkat cells or might not be involved in the etoposide-induced DNA fragmentation. Since the inhibition of caspases 3, 8 and 9 is accompanied by the down regulation of the activity of a cascade of caspases (caspases 2, 6, 7, 9 and 10), selectivity of caspase-I inhibitors can be

ascertained for the above panel (caspases 2, 6, 7, 8, 9 and 10) of caspases from this single assay.

Keywords Caspases · Apoptosis · DNA fragmentation · IL-1 β · Rheumatoid arthritis · Inflammation

Introduction

Apoptosis is an evolutionarily conserved phenomenon of cell suicide that follows a specialized cellular process. A cascade of proteolytic enzymes called caspases is the central component of this process, which result in the cleavage of protein substrates, resulting in disassembly of the cell (Thornberry and Lazebnik 1998). Inappropriate apoptosis is a prominent feature of many human diseases, making caspases as attractive targets for therapeutic interventions (Wu and Fritz 1999).

The mammalian Caspase family is involved in apoptosis induced by many types of stimuli such as anti-Fas antibody and chemical inducers such as etoposide (Sun et al. 1999). Current evidences suggest that apoptosis involves a sequentially activated cascade of caspases (Muzio et al. 1996) with the involvement of at least 14 members of this family (Van de Craen et al. 1998). However, the precise role and contribution of individual caspases are still not fully

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understood. Caspases are synthesized as pro-enzymes and activated by cleavage at specific aspartate residues. Cleaved caspases are responsible for the biochemical and morphological changes associated with the apoptosis in the affected cells (Cohen 1997). Caspases are classified into several classes; the initiator caspases (caspases 2, 8, 9 and 10), which activate the downstream executioner caspases (caspases 3, 6, and 7), and are responsible for cleaving intracellular substrates, such as poly (ADP-ribose) polymerase (PARP) and laminin, resulting in the disassembly of the cell (Cohen 1997; Kidd 1998). One more distinct class of Caspases, designated as ICE (Interleukin Converting Enzymes) subfamily, comprising of caspases-1, -4, -5 and -13 (Ashwell 2001) is mainly involved in cytokine maturation, leading to an inflammatory response. The main substrate of this family is IL-1 β , a key signaling protein involved in inflammation, and therefore inhibition of this family of caspases might be a valuable treatment for inflammatory diseases such as rheumatoid arthritis (Iwamura 2000).

A large number of approaches have been used to measure caspase activity in vitro. These include, cleavage of caspase specific substrates using specific purified caspases enzyme, which can be detected either by fluorescence or by colorimetric assays. An alternative method includes measuring the activity of caspases by immunoblotting. This method is based on the detection of caspase cleavage products, which differ in molecular weight from the pro-caspases, using caspase-specific antibodies (Zhivotovsky et al. 1999). This approach has been extended to measure activation of caspases in situ using antibodies specific for active caspases that do not cross-react with pro-caspases. Alternatively, a specific antibody against a cleavage product of the caspases such as, poly (ADP-ribose) polymerase can be used to determine the activation of caspases (Li and Darzynkiewicz 2000). Altogether, these methods have been very helpful for fundamental research during the last 10 years, and in particular for the identification of the molecular mechanisms involved in caspase activation. However, most of these assays require either specific antibodies or additional reagents for measuring enzyme activity, making these assays costly and time consuming. Therefore, there is a need to develop a fast, reliable, cost effective and robust assay.

In the light of these observations, the aim of the present study is to develop a cell-based method to

determine the caspase activity in biologically relevant context. Here, using etoposide-induced apoptosis of Jurkat cell line, we describe a novel assay to screen inhibitors of ICE family of caspases (1, 4 and 5) from rest of the caspases (6, 7, 8, 9, 10 etc.). This cell-based assay can be used as a secondary assay in combination with a caspase-specific enzyme assay.

Materials and methods

Materials

RPMI-1640 medium and bovine serum albumins were purchased from Invitrogen. Etoposide was purchased from Sigma St. Louis, MO. Caspases inhibitors (YVAD-CHO, DEVD-CHO, IETD-CHO, LEHD-CHO and Z-VAD-FMK), caspase substrates (Ac-YVAD-pNA, Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA) and recombinant caspases enzymes (Caspases-1, -3, -8 and -9) were purchased from Calbiochem. YN-1234, (N-2-naphthoyl-L-valyl-N-[(1S)-2-carboxy-1-formylethyl]-L-prolinamide), a Yamanouchi compound, was synthesized in-house.

Cell culture

Jurkat cells, a human T-lymphocyte cell line, were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine (complete medium) in an atmosphere of 5% CO₂ at 37 °C.

Caspase-1, -3, -6, -7, -8, -9 and -10 enzymatic assays

The activity of caspases 1, 3, 4, 6, 7, 8, 9 and 10 was determined using a colorimetric protease assay. This assay is based on the ability of the active enzyme to cleave the chromophore pNA from the enzyme-specific substrates, Ac-YVAD-pNA (caspase-1, -4 and -5), Ac-DEVD-pNA (caspase-3, -6, -7, -8 and -10), Ac-IETD-pNA (caspase-8) and Ac-LEHD-pNA (Caspase-9). Finally, the absorbance was measured at 405 nM. Assays were performed in 2 $\times$ Reaction buffer (100 mM HEPES [pH-7.2], 100 mM NaCl, 0.2% CHAPS, 20 mM EDTA, 10% Glycerol and 10 mM DTT) and were initiated in 96 well plate (total reaction volume was 100 μ L/well) by addition

of human recombinant Caspases (1, 3, 8 and 9), diluted in assay buffer to 0.2 units/ μ L, with the appropriate dilutions of inhibitor in DMSO and assay buffer. The plate was then incubated on rotatory shaker for 10 min and at the end of incubation period, appropriate substrates (200 μ M) were added for the respective caspases. The plate was then incubated at 37 °C for 1 h and finally absorbance was read at 405 nm using a microplate ELISA reader.

In experiments with caspases inhibitors, recombinant enzymes were pre-incubated with specific inhibitors of various caspases (YVAD-CHO, DEVD-CHO, IETD-CHO, LEHD-CHO and Z-VAD-FMK) (100 μ M each) for 10 min before the addition of substrate.

Measurement of caspase-1, -3, -6, -7, -8, -9 and -10 enzymatic activity in the cell lysate of Jurkat cells

Caspase activity in the crude cell lysate of Jurkat cells was also determined as described above for the recombinant enzymes. In brief, Jurkat cells were seeded at a density of 2×10^6 cells/mL in six well plates in an atmosphere of 5% CO₂ at 37 °C. Cells were stimulated by incubating with etoposide (68 μ M) for 18 h. In experiments with caspases inhibitors, cells were pre-incubated with inhibitors (100 μ M each) for 1 h before induction with etoposide for additional 18 h. Etoposide treated and non-treated Jurkat cells (with and without specific inhibitors of various caspases) were centrifuged at 3,000 rpm for 5 min and cell pellets were lysed in lysis buffer (10 mM/L Tris, [pH 7.4], 1 mM/L dithiothreitol, 2 mM/L EDTA, 1 mM/L PMSF, 10 mg/mL pepstatin and 10 mg/mL leupeptin) for 30 min on ice. After centrifugation at 14,000 rpm for 30 min at 4 °C, the supernatant was used to assess caspase activity after determining the concentration of protein by Bradford protein assay kit. The proteolytic reaction was carried out in 2 $\times$ reaction buffers containing 50 μ g of cytosolic protein extracts and Ac-YVAD-pNA, DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA at a concentration of 200 μ M in the separate reaction mixtures (total reaction volume was 100 μ L/well) in a 96 well plate. The reaction mixture was incubated for 1 h at 37 °C and finally absorbance was measured at 405 nm using a microplate ELISA reader.

DNA fragmentation assay

Fragmented DNA was isolated as per the protocol described earlier with slight modifications (Tafani et al. 2000). In brief, Jurkat cells were seeded in a density of 2×10^6 cells/mL in six well plates in an atmosphere of 5% CO₂ at 37 °C. Cells were stimulated by incubating with etoposide (68 μ M) for 18 h. Etoposide treated and un-treated Jurkat cells (with and without caspases inhibitors) were centrifuged at 3,000 rpm for 5 min. The cell pellet was washed twice in PBS and then lysed in 250 μ L of lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA and 0.5% Triton X-100) on ice for 30 min. The lysate was centrifuged at 13,000 rpm for 30 min at 4 °C. RNase (0.2 mg/mL) was added, and the lysate was incubated for 30 min at 37 °C. Proteinase K (0.1 mg/mL) and SDS (final concentration 1%) were added, followed by incubation at 56 °C for 2 h. DNA was extracted with phenol/chloroform and then with chloroform, precipitated with ethanol and sodium acetate, and electrophoretically separated (Using a constant voltage of 80 Volt) on a 1.2% agarose gel containing 1 μ g/mL ethidium bromide and visualized under ultraviolet transillumination.

Results and discussion

Caspases belong to the family of ICE proteases, which play critical roles in both cytokine maturation and apoptosis. These caspases are believed to trigger both the onset and resolution of inflammation by regulating the maturation of IL-1 β , a major cytokine that control inflammatory and immune responses. Hence, there has been increasing interest in the pharmacological modulation of caspases, which may provide rational therapies for inflammatory diseases, such as rheumatoid arthritis, or in neurodegenerative disorders and ischemia-related cell death (Ashwell 2001). In spite of the earlier failure in the development of inhibitors, caspases are still an attractive target for intervention.

Major concerns of all drug discovery programs are selectivity of drugs; caspase inhibitors are also not an exception to this. Therefore, selection of a caspase assay is a daunting task, as the assay should be cost effective, flexible and less time consuming.

Cell-based assays inherently evaluate the activity of the test compound in a biologically relevant context, with the added potential for extraction of high information content in virtually every therapeutic area.

Caspases generally work in a cascade, in which signal captured by a particular Caspase is passed on to a number of downstream caspases, before being translated into a particular pathway, such as apoptosis. In such cascades, final outcome is a result of

contribution made by each component of that pathway. Therefore, cell based assays designed to monitor the activation or function of important biological pathways can be an assay of choice for primary or selectivity screening.

Human T-cell leukemia cell line, Jurkat has been used to examine the activation of different caspases in response to various stimuli. In a study using Northern blotting, it has been shown that full-length message was detected for procaspases-2, -3, -6, -7, -8,

Fig. 1 Evaluation of Caspase-1, -4, -6, -7, -8, -9 and -10 activity in the etoposide treated Jurkat cell lysate. Jurkat cells were pre-incubated for 1 h with YVAD-CHO, DEVD-CHO, IETD-CHO, LEHD-CHO and Z-VAD-Fmk (100 μ M) each in different wells of six wells culture plate. Cells were then incubated with etoposide (68 μ M) for additional 18 h. Cells were lysed, concentration of protein was determined and proteolytic reaction was setup using caspases specific substrate. The reaction mixture was incubated for 1 h and absorbance was measured at 405 nM. **A** Evaluation of Caspase-1, -4 and -5

activities. Caspase-1, -4 and -5 activity was measured in Jurkat cell lysate using caspase-1, -4 and -5 specific substrate (Ac-YVAD-pNA). **B** Evaluation of Caspase 3, 6, 7 and 10 activities. Activity of Caspase-3, -6, -7 and -10 was measured in Jurkat cell lysate using Ac-DEVD-pNA, a common substrate for all these caspases. **C** Evaluation of Caspase 8 activity. Caspase-8 activity was measured in Jurkat cell lysate using caspase-8 specific substrate (Ac-IETD-pNA). **D** Evaluation of Caspase 9 activity. Caspase-9 activity was measured in Jurkat cell lysate using caspase-9 specific substrate (Ac-LEHD-pNA)

and -10 in Jurkat cells. In contrast, levels of mRNA for procaspases-1, -4, and -5 were below the detectable limit (Eischen et al. 1997). Jurkat cells treated with FTY720, a potent immunosuppressive agent and inducers of apoptotic cell death, showed activation of Caspases 2, 3, 6, 8, 9 and 10 in a time-dependent manner and also exhibited various features of apoptosis (Fujino et al. 2001). However, caspases 1 and 5 were not activated.

With this background, we checked the presence of different caspases in the cell lysate of Jurkat cells that were untreated or treated with etoposide. Cell lysate of untreated Jurkat cells showed presence of caspases-3, -6, -7, -8, -9 and -10 and in etoposide treated Jurkat cells, activity of these caspases increased significantly (Fig. 1B–D). However, caspase 1, 4 and 5 activity was not observed in untreated or etoposide treated cells (Fig. 1A). Inhibitors of caspases 3, 6, 7, 8 and 10 (DEVD-CHO), caspase 8 (IETD-CHO), caspase 9 (LEHD-CHO), and a broad-spectrum caspase inhibitor (Z-VAD-FMK) prevented etoposide induced activation of specific caspase activity (Fig. 1B–D), whereas the caspase 1, 4 and 5 inhibitor (YVAD-CHO) did not show any inhibition (Fig. 1A). In an earlier study, treatment of Jurkat cells with etoposide resulted in proteolytic cleavage of precursors for the caspase-3, -8 and -9 (Fujino et al. 2002). The specific inhibitor of caspase-3 (DEVD-CHO) and broad caspases inhibitors, Z-VAD-FMK, and Z-Asp-CH2-DCB prevented etoposide mediated apoptosis; however, specific inhibitor of caspase-1 (YVAD-CHO) failed (Fujino et al. 2002). These observations collectively confirmed that caspases belonging to ICE family (Caspases 1, 4 and 5) are absent in Jurkat cells and etoposide treatment of Jurkat cells activates caspases, 3, 6, 7, 8, 9 and 10 (Fig. 1).

Subsequently, we investigated whether etoposide induced cell death is through apoptosis. DNA fragmentation assay revealed that incubation of cells with etoposide resulted in the formation of DNA ladder, a hallmark of apoptosis, confirming apoptotic nature of the cell death (Fig. 2). We then evaluated the role of individual caspase inhibitors on etoposide-induced apoptosis. We observed etoposide-induced apoptosis in Jurkat cells, as evident by the formation of DNA fragments (Fig. 2A, lane 4). However, pre-incubation of Jurkat cells with caspases 3, 6, 7, 8, 9, 10 and PAN caspase inhibitor resulted in the significant inhibition of etoposide-induced DNA fragmentation, whereas,

Fig. 2 Etoposide mediated DNA fragmentation in Jurkat cells. Jurkat cells were pre-incubated with caspase inhibitors-YVAD-CHO, DEVD-CHO, IETD-CHO, LEHD-CHO, Z-VAD-FMK and YN-1234 (100 μ M each) for 1 h. Cells were then incubated in the presence of 68 μ M/mL etoposide for 16 h. DNA from the etoposide treated and untreated cells (with or without pre-incubation with caspases specific inhibitors) were then subjected to agarose gel electrophoresis. Agarose gels shown in (A, B) are two different gels run on same day and under similar electrophoresis conditions. **A** Lane 1: DNA ladder; lane 2: Untreated cells; lane 3: Etoposide treated with LEHD-CHO; lane 4: Etoposide only; lane 5: Etoposide treated with DMSO (1%); lane 6: Etoposide treated with YVAD-CHO; lane 7: Etoposide treated with DEVD-CHO; lane 8: Etoposide treated with IETD-CHO. **B** Lane 1: DNA ladder; lane 2: Untreated cells; lane 3: Etoposide only; lane 4: Etoposide treated with Z-VAD-FMK; lane 5: Etoposide treated with YN-1234

selective inhibitor of ICE family of caspases did not show any effect on etoposide-induced DNA fragmentation (Fig. 2). These results collectively

Table 1 In vitro profile of caspase inhibitors

Compound	Caspase-1 assay (IC ₅₀)	Caspase-3 assay (IC ₅₀)	Caspase-8 assay (IC ₅₀)	Caspase-9 assay (IC ₅₀)
YN-1234	24 nM	>30 μ M	224 nM	3.4 μ M
YVAD-CHO	10 nM	ND	ND	ND
DEVD-CHO	ND	5.0 nM	ND	ND
IETD-CHO	ND	ND	10 nM	ND
LEHD-CHO	ND	ND	ND	13 nM

ND not done

confirmed, that etoposide induced apoptosis is mediated by Caspases 3, 8, 9, 10 and their downstream caspases, whereas Caspases-1, -4 and -5 have no role to play. These results were corroborated by earlier study in which, inhibitors of caspase 3 (DEVD-CHO), caspase 8 (IETD-CHO), caspase 9 (LEHD-CHO), and a broad-spectrum caspase inhibitor (Z-Asp-CH-DCB) prevented FTY720 induced DNA fragmentation, while the caspase 1 inhibitor failed (Li and Darzynkiewicz 2000).

To validate these results, we used Caspase-1 inhibitor, YN-1234 of Yamanouchi Pharmaceutical Co. (Okamoto et al. 1999), synthesized in-house. In the enzyme assay, YN-1234 (N-2-naphthoyl-L-valyl-N-[(1S)-2-carboxy-1-formylethyl]-L-prolinamide) inhibited human recombinant Caspase-1 with an IC₅₀ of 24 nM. Similarly, Table 1 suggests that YN1234 inhibits Caspase-8 with an IC₅₀ value of 224 nM, however, this compound did not inhibit caspase 3 and 9 upto a potency of 100-fold compared to its potency for caspase-1 inhibition. This data collectively suggests that YN1234 is a Caspase 1 selective inhibitor, which non-selectively inhibits caspase 8, but spares caspase 3 and 9 (Table 1). As per our hypothesis any compound that will inhibit caspases other than the caspases belonging to the ICE family of caspases, will inhibit etoposide induced DNA fragmentation.

To test this hypothesis, we checked the effect of YN1234 on etoposide induced DNA fragmentation. We observed that YN1234, unlike caspase-1 selective inhibitor (YVAD-CHO) (Lane 6, Fig. 2A), showed inhibition of etoposide-induced DNA fragmentation in Jurkat cells (Lane 5, Fig. 2B). Since YN1234 also inhibits Caspase 8, it was expected to inhibit etoposide-induced DNA fragmentation in Jurkat cells, similar to caspase 8 inhibitor (IETD-CHO), lane 8 Fig. 2A. Since inhibition of DNA fragmentation is accompanied by down regulation of the activity of cascade of caspases such as caspases, 3,

6, 7, 8, 9 and 10, therefore, YN-1234 might be down regulating the activity of any of these caspases. Therefore, results of the apoptotic assay confirmed our enzyme assay results that YN-1234 is not selective for caspase-1 family.

This study provides a new cell-based screening method for identifying caspase-1 selective compound, as any compound that inhibits the activity of caspases except for ICE family of caspases will inhibit the etoposide induced apoptosis in Jurkat cells. We have tested several compounds from our compound library using this method and validated these results using a colorimetric enzyme assay. We found that both assays showed a good correlation (data not shown). These studies confirm that the proposed cell-based assay can be conducted in combination with the colorimetric reference method to evaluate the selectivity profile of Caspase inhibitors. This study will certainly expedite research in the field of finding caspase-1 selective inhibitors, as no convenient assay suitable for large-scale selectivity screening is available against a panel of multiple Caspases. The major advantage of this assay is that it is robust and cost effective, as compared to other available assays that need costly reagents including purified recombinant enzymes and/or antibodies. This assay meets additional prerequisite factors such as data quality and throughput, needed to be taken into consideration for developing an assay for screening compound libraries. Clearly, the cell-death based assay presented in this report offers attractive advantages for both fundamental and pharmaceutical researchers.

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