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Characterization of a Novel and Specific Inhibitor for the Pro-apoptotic Protease Omi/HtrA2*

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Omi/HtrA2 is a mammalian serine protease with high homology to bacterial HtrA chaperones. Omi/HtrA2 is localized in mitochondria and is released to the cytoplasm in response to apoptotic stimuli. Omi/HtrA2 induces cell death in a caspase-dependent manner by interacting with the inhibitor of apoptosis protein as well as in a caspase-independent manner that relies on its protease activity. We describe the identification and characterization of a novel compound as a specific inhibitor of the proteolytic activity of Omi/HtrA2. This compound (ucf-101) was isolated in a high throughput screening of a combinatorial library using bacterially made Omi-(134–458) protease and fluorescein-casein as a generic substrate. ucf-101 showed specific activity against Omi/HtrA2 and very little activity against various other serine proteases. This compound has a natural fluorescence that was used to monitor its ability to enter mammalian cells. ucf-101, when tested in caspase-9 (−/−) null fibroblasts, was found to inhibit Omi/HtrA2-induced cell death.

Omi/HtrA2 is a human serine protease that has extensive homology to bacterial high temperature requirement A (HtrA)[1] proteins (1, 2). Bacterial HtrAs have a dual function, acting as chaperones at normal temperatures and as proteases at elevated temperatures and removing damaged or denatured proteins, allowing the recovery and survival of bacteria following stress (3, 4). Mammalian Omi/HtrA is a ubiquitous protein, although tissue-specific alternatively spliced forms have been reported (2, 5). Recent studies (6–10) described Omi as a mitochondrial protein that upon induction of apoptosis is released to the cytoplasm where it binds XIAP (X chromosome-linked inhibitor of apoptosis protein) resulting in caspase-9 activation. In this regard, Omi resembles Smac/DIABLO, which also binds IAPs and as a result activates caspase-9 (11, 12). Omi protein is synthesized as a precursor that is processed in the mitochondria to produce the mature protein. This processing exposes an internal tetrapeptide motif, AVPS, at the amino terminus. A similar motif is found in all IAP-binding proteins, including the *Drosophila* Grim, Reaper, Hid, and Sickle (13–16). Processing of the precursor Omi polypeptide is an intramolecular reaction and requires an intact protease domain.[2] Omi can also induce apoptosis in a caspase-independent pathway that relies entirely on its ability to function as a serine protease (6, 7, 9). Neither the mechanism nor the significance of this function of Omi is clearly understood. In order to investigate the caspase-independent mechanism of apoptosis initiated by Omi, we isolated specific inhibitors of its proteolytic activity. One such family of synthetic compounds was identified and is described in this paper. These heterocyclic compounds showed significant and specific activity against the protease in an *in vitro* assay. A compound from this family, which exhibited the highest activity against Omi, was selected for more detailed studies. This compound (ucf-101) naturally fluoresces and easily enters mammalian cells, allowing its use in *in vivo* experiments. Our results clearly showed ucf-101 had a profound effect on the activity of Omi and could substantially inhibit its ability to induce caspase-independent apoptosis in caspase-9 (−/−) null fibroblasts (17).

The ucf-101 inhibitor can be used as a tool to dissect the two different activities (caspase-dependent *versus* caspase-independent) of Omi and their respective contribution to apoptosis in various biological settings. Furthermore, ucf-101, or a similar compound, may be useful as an anti-apoptotic drug that would specifically target caspase-independent cell death under clinical conditions.

EXPERIMENTAL PROCEDURES

Preparation of FITC-labeled, Dephosphorylated Casein—Dephosphorylated-casein (Sigma, 3 ml of 2 mg/ml stock solution) was used in a dialysis cassette (Pierce) placed in labeling buffer (0.03 mg/ml FITC, 50 mM sodium borate, pH 9.3, 40 mM NaCl) and kept for 48 h at 4 °C in the dark with constant stirring. After labeling, the FITC-dephosphorylated casein (Dcasein-FITC) was further dialyzed against 50 mM Tris-HCl, pH 7.5, and 50 mM NaCl to remove any residual unlabeled FITC. The Dcasein-FITC conjugate was recovered from the dialysis cassette and stored at 4 °C in the dark.

Expression and Purification of MBP-Omi and MBP-L56 Proteases—PCR was used to amplify DNA sequences corresponding to Omi/HtrA2 (amino acids 134–458) and L56/HtrA1 (amino acids 140–480). The PCR products were digested with *Mfe*I and *Xba*I and cloned in the

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[1] The abbreviations used are: HtrA, high temperature requirement A; IAP, inhibitor of apoptosis protein; XIAP, X chromosome-linked inhibitor of apoptosis protein; t-PA, tissue plasminogen activator; urokinase-type plasminogen activator; FITC, fluorescein isothiocyanate; MBP, maltose-binding protein; GFP, green fluorescent protein.

[2] L. Cilenti, A. Yerkes, L. Musumeci, and A. Zervos, submitted for publication.

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TABLE I
Screening for inhibitors of the proteolytic activity of Omi

A combinatorial library of 528 selected compounds was used in a high throughout screening for potential inhibitors of the proteolytic activity of Omi. Several compounds had weak activity against Omi, but after two rounds of selection only one chemical showed substantial and reproducible inhibition against Omi in the assay used.

% inhibition	1st screening	2nd screening
0	6	26
1–19	468	47
20–39	52	1
40–59	2	1
Total	528	75

corresponding *Eco*RI and *Xba*I restriction sites of pMAL-p2X vector (New England Biolabs). MBP-Omi-(134–458) and MBP-L56-(140–480) proteases were expressed in TB1 *Escherichia coli* (New England Biolabs) and purified on an amylose binding affinity column as described by the manufacturer (New England Biolabs). The concentration of purified proteases was determined using the Bradford assay.

Expression and Purification of His-tagged Omi-(134–458)—PCR was used to amplify the DNA sequence corresponding to Omi/HtrA2 (Omi-(134–458)). The PCR product was cloned in-frame in the bacterial expression vector pET-28 (Novagen). For bacterial expression, BL21 (DE3) (Novagen) bacteria were transformed with pET-Omi-(134–458). Single colonies were grown overnight in LB medium containing kanamycin. The overnight culture (1 ml) was used to inoculate 1 liter of LB medium, and growth was continued at 37 °C until the A_{600} was ~0.8. At this time, 1 mM isopropyl-1-thio-β-D-galactopyranoside was added, and the culture was placed in a shaking incubator overnight at 20 °C. Bacteria were harvested by centrifugation and lysed in a buffer containing 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 100 μg/ml lysozyme, and a protease inhibitor mixture (Sigma). The bacterial suspension was then sonicated, and the soluble fraction was purified using nickel-nitrilotriacetic acid His-Bind resin (Novagen). The purity of the His-Omi-(134–458) protein was monitored by SDS-PAGE and Coomassie Blue staining of the resulting gel.

Proteolytic Activity of Omi Using Dcasein-FITC as Substrate—Opaque microtiter plates (Corning Glass) were used in order to minimize background absorbance. A typical assay included 10 μg of Dcasein-FITC substrate, 2 μg of MBP-Omi-(134–458) in assay buffer (20 mM Na_2HPO_4/NaH_2PO_4, pH 7.4, 200 mM NaCl, 5% glycerol) in a final volume of 100 μl. Dcasein-FITC conjugate solution (50 μl of a 0.2 μg/μl stock solution) was added in each well using a Multidrop 384 multiple dispenser (Labsystems) and incubated in the Wallac 1420 Victor^2 Multilabel Counter at 37 °C for 15 min. After this time, 50 μl of MBP-Omi-(134–458) protease (40 ng/μl) was added to each well. The fluorescence (535 nm) of the reactions was recorded every 5 min for a 30-min period.

Combinatorial Library Screening—A Pharma Library Collection (Nanosyn) was screened for compounds, which may inhibit the proteolytic activity of MBP-Omi-(134–458) in an *in vitro* assay. Each compound, 10 μM final concentration, was preincubated with 50 μl of MBP-Omi-(134–458) (2 μg) for 15 min at 37 °C. After this time, Dcasein-FITC solution (10 μg) was added, and the change in fluorescence was read every 5 min during a 30-min period using a 535-nm bandpass filter. The proteolytic activity of MBP-Omi-(134–458) was expressed as the percentage inhibition, where 100% refers to the activity in the absence of inhibitor (Me_2SO replaced the inhibitor in the assay).

Activity of Several ucf Analog Compounds Against MBP-Omi-(134–458)—The assay was performed as described above using Dcasein-FITC as substrate. 2 μg of MBP-Omi-(134–458) was incubated with various concentrations of ucf-101, ucf-102, ucf-103, or ucf-104 for 15 min. After this time 10 μg of Dcasein-FITC substrate was added, and the reaction proceeded for an additional 30 min. The activity of MBP-Omi-(134–458) was monitored at 535 nm.

Determination of IC_{50} for ucf-101 and ucf-102—The IC_{50} value for each of the two selected inhibitors was obtained using His-Omi-(134–458) and the EnzCheck assay kit (Molecular Probes) that contains BODIPY FL-casein as a generic substrate. Briefly, 35 μl of His-Omi-(134–458) (500 nM) diluted in 20 mM Na_2HPO_4/NaH_2PO_4, pH 7.4, 200 mM NaCl, 5% glycerol was incubated with 5 μl of various concentrations of the inhibitors (0.1–1000 μM) in 100% Me_2SO (final concentration of Me_2SO was 10%); 10 μl of BODIPY-FL casein (2.5 μM final concentration) diluted in buffer was added. The assay was carried out using 384-well microtiter plates. Fluorescence was monitored continuously for 10 min at 37 °C on a Tecan SpectraFluorPlus microtiter plate reader

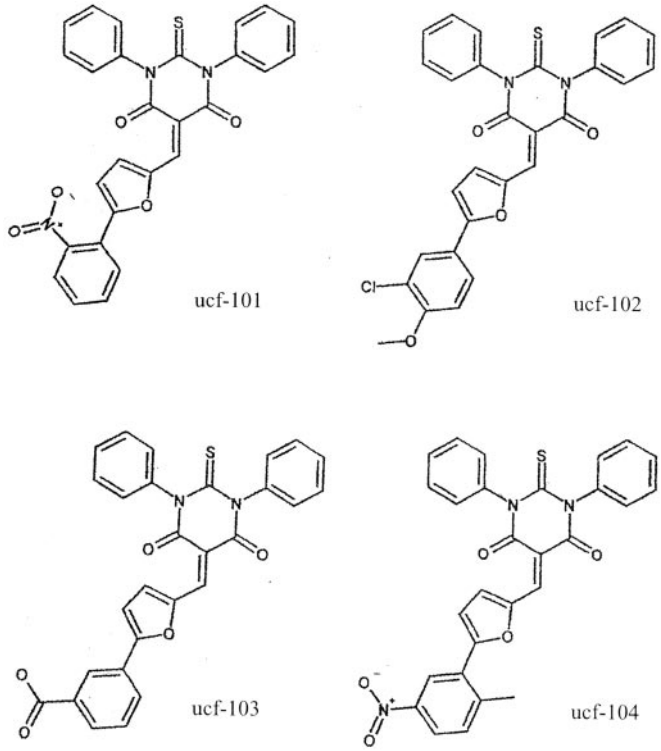

FIG. 1. **Chemical structures of ucf-101, ucf-102, ucf-103, and ucf-104.**

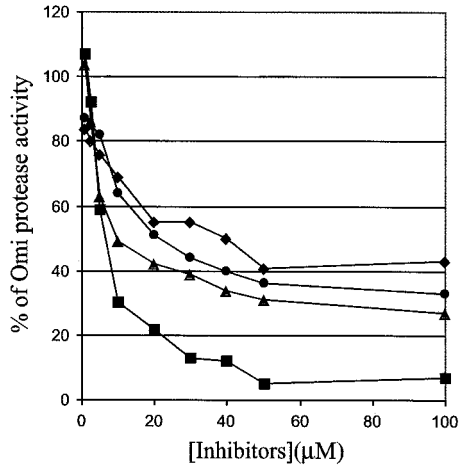

FIG. 2. **Inhibition of MBP-Omi-(134–458) by ucf-101 and several other ucf analogs.** The assay was performed with 10 μg of Dcasein-FITC incubated with 2 μg of MBP-Omi-(134–458) in the presence of various concentrations of ucf-101 and three analogs, ucf-102, ucf-103, and ucf-104. ■, ucf-101; ▲, ucf-104; ●, ucf-103; ◆, ucf-102.

(Tecan, Crailsheim, Germany) at an excitation wavelength 485 nm/emission wavelength 530 nm. IC_{50} values were calculated using the GraFit 4 program (Erithacus Software, Middlesex, UK).

Activity of ucf-101 against Various Serine Proteases—The amount of the inhibitor giving a 50% (IC_{50}) decrease of the enzyme activity compared with the control reaction was estimated for various serine proteases. Briefly, 35 μl of the indicated enzyme diluted in the corresponding buffer was incubated for 10 min with 5 μl of various concentrations of inhibitor (0.1–1000 μM) in 50% Me_2SO, 50% buffer. After this time, 10 μl of the corresponding peptidic colorimetric or fluorogenic substrate diluted in buffer was added. In the case of the FVIIa/TF assay, a preincubation of both proteins for 10 min at room temperature was performed prior to addition of the inhibitor to allow complex formation. For this experiment the following materials were used: canine FXa, rat FXa, and rabbit FXa (Enzyme Research Laboratories); trypsin from bovine pancreas and human thrombin (Sigma); human plasmin, human t-PA, human u-PA, and human kallikrein (Calbiochem-Novabiochem);

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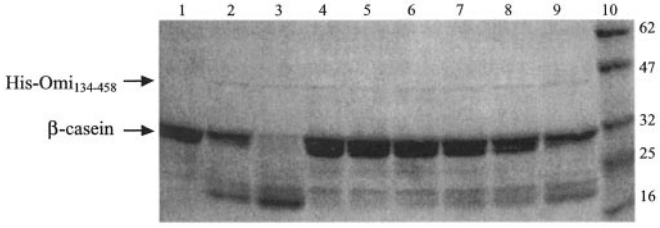

FIG. 3. ***In vitro* assay of the proteolytic activity of His-Omi-(134–458) in the presence of different concentrations of ucf-101.** The assay was performed at 37 °C, and after the indicated time points the reactions were resolved on SDS-PAGE and the gel stained with Coomassie Blue. Omi (200 ng) was incubated with 5 μg of β-casein substrate in a 20-μl reaction volume for 30 min at 37 °C. The ucf-101 compound was preincubated with His-Omi-(134–458) (His-Omi) protease for 10 min at room temperature prior to the addition of β-casein substrate. *Lane 1*, β-casein control; *lane 2*, His-Omi + β-casein, digested for 15 min; *lane 3*, His-Omi + β-casein, digested for 30 min; *lane 4*, His-Omi + 100 μM ucf-101 + β-casein; *lane 5*, His-Omi + 80 μM ucf-101 + β-casein; *lane 6*, His-Omi + 50 μM ucf-101 + β-casein; *lane 7*, His-Omi + 30 μM ucf-101 + β-casein; *lane 8*, His-Omi + 20 μM ucf-101 + β-casein; *lane 9*, His-Omi + 10 μM ucf-101 + β-casein; and *lane 10*, prestained molecular weight marker.

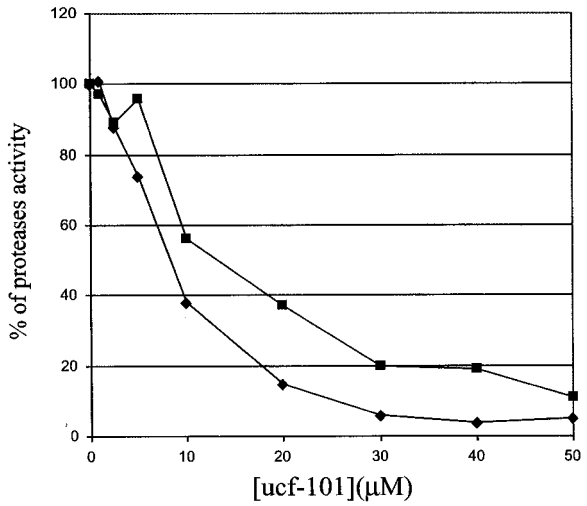

FIG. 4. **Inhibition of the proteolytic activity of MBP-Omi-(134–458) and MBP-L56-(140–480) by ucf-101.** The assay was performed with 10 μg of FITC-Dcasein and 2 μg of MBP-Omi-(134–458) (MBP-Omi) or MBP-L56-(140–480) (MBP-L56) in the presence of various concentrations of ucf-101. The increase in fluorescence for the initial 20 min was used for comparing the activities of MBP-Omi-(134–458) or MBP-L56-(140–480). ◆, MBP-Omi; ■, MBP-L56.

human APC and recombinant human factor VIIa (American Diagnostica); and recombinant tissue factor (Dade Behring). The substrates used are as follows: spectrozyme fXa fluorogenic (American Diagnostica); kallikrein fluorogenic substrate; protein C-activated substrate (Calbiochem); Chromozym X, Chromozym PL, and Chromozym t-PA (Roche Diagnostics); I-1045, I-1140, and I-1140 (Bachem).

The amount of each enzyme and its corresponding substrate used in the assay was canine FXa (2 nM), spectrozyme fXa fluorogenic (20 μM), rat FXa (5 nM), spectrozyme fXa fluorogenic (25 μM), rabbit FXa (5 nM), spectrozyme fXa fluorogenic (15 μM), kallikrein (5 nM), kallikrein fluorogenic substrate (50 μM), trypsin (2.5 nM), Chromozym X (150 μM), plasmin (5 nM), I-1045 (100 μM), thrombin (4 nM), Chromozym PL (150 μM), FVIIa/tissue factor (15 nM/3 nM), Chromozym t-PA (500 μM), t-PA (100 nM), I-1140 (200 μM), u-PA (3 nM), I-1140 (150 μM), activated protein C (5 nM), and protein C-activated substrate (100 μM) in a final concentration of 5% Me_2SO. All enzymatic assays were carried out at room temperature in 384-well microtiter plates (Nunc). Color development due to the release of *p*-nitroanilide from the chromogenic substrates was monitored continuously for 20 min at 405 nm on a Tecan SpectraFluorPlus microtiter plate reader (Tecan). Fluorescence from the release of the coumarin derivative, aminomethylcoumarin, was measured at excitation 360 nm/emission 465 nm on the same reader. The IC_{50} values were calculated using the GraFit 4 program (Erithacus Software).

TABLE II
IC_{50} of ucf-101 and ucf-102 inhibitors on His-Omi
The value is the average from three independent experiments.

Inhibitors	IC_{50} in μM
ucf-101	9.5
ucf-102	45.9

TABLE III
IC_{50} values of ucf-101 on various proteases
The two IC_{50} values are derived from two independent experiments.

Protease	IC_{50} in μM
Canine-FXa	430
	360
Rat-FXa	410
	>500
Rabbit-FXa	260
	300
Hu-kallikrein	200
	250
Bovine-trypsin	>500
	>500
Hu-plasmin	>500
	400
Hu-thrombin	>500
	>500
Hu-rec.-FVIIa	>500
	>500
Hu-t-PA	390
	390
Hu-u-PA	500
	500
Hu-APC	500
	500

Subcellular Localization of ucf-101—HeLa cells were grown on coverslips using F-12 (Ham's) nutrient mixture (Invitrogen) supplemented with 10% fetal calf serum (Sigma), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Different concentrations of ucf-101 were added to the cell media, and after 2 h cells were washed three times with phosphate-buffered saline and fixed with 4% paraformaldehyde. The coverslips were then placed on glass slides using Fluoromount-G solution (Southern Biochemical Association). The subcellular localization of ucf-101 was monitored using a LSM510 confocal laser-scanning microscope (Zeiss).

ucf-101 Inhibits Omi-induced Caspase-independent Apoptosis in Caspase-9 (−/−) Null Fibroblasts—Caspase-9 (−/−) cells were transfected with either pEGFP-N1 vector (Clontech) or M-Omi-GFP that encodes a cytoplasmic form of Omi/HtrA2 (7). Transfected cells were kept in medium containing different concentrations of ucf-101. This medium, including the inhibitor, was replaced every 12 h. After 36 h, cells were stained with propidium iodide and 4′,6-diamidino-2-phenylindole as described (7). Normal and apoptotic GFP-expressing cells were counted using fluorescence microscopy.

RESULTS

Activity of Omi Protease against Dcasein-FITC—Because no physiological substrates for Omi protease are known, β-casein was used as a generic substrate in *in vitro* assays to monitor the protease activity of Omi. For high throughput screening, we developed a new assay that uses a mixture of α-casein, β-casein, and dephosphorylated casein (Dcasein) coupled to FITC, as a substrate. The use of Dcasein-FITC increases the fluorescence during the reaction with less fluctuation and lower background (data not shown). A typical assay uses 10 μg of Dcasein-FITC and 2 μg of MBP-Omi-(134–458) in 100 μl of reaction buffer (20 mM sodium phosphate buffer, pH 7.5, 200 mM NaCl, and 5% glycerol) at 37 °C for 30 min. There was a rapid increase in fluorescence for the first 20 min followed by a slower

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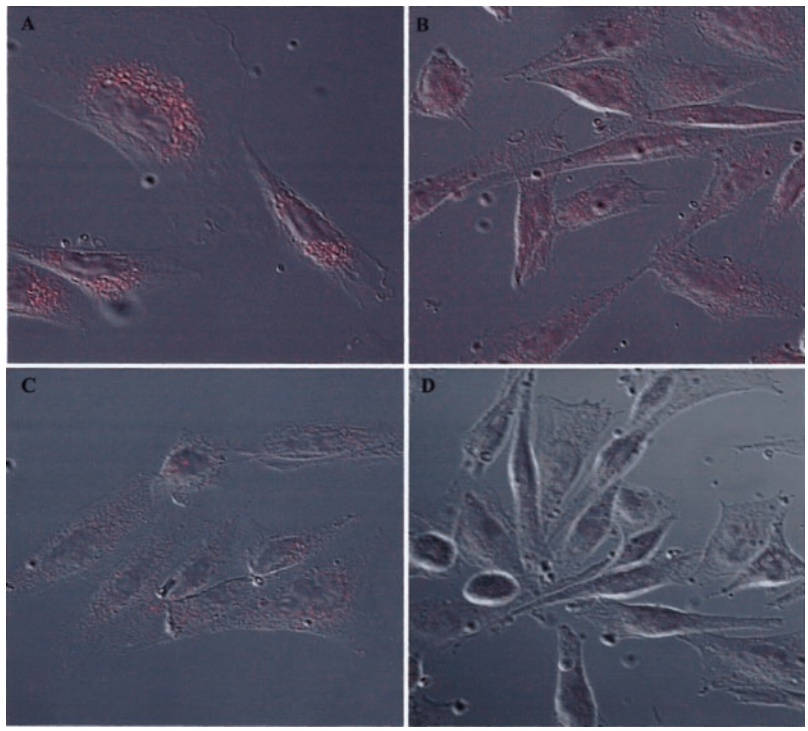

FIG. 5. **Cytoplasmic localization of ucf-101.** Subconfluent HeLa cells were treated with 50 (*A*), 40 (*B*), and 20 μM (*C*) ucf-101 and were observed using a confocal microscope. *A–C* show cytoplasmic red staining due to the presence of ucf-101, indicating cells are permeable to this chemical. *D*, no staining was seen when Me_2SO alone was used.

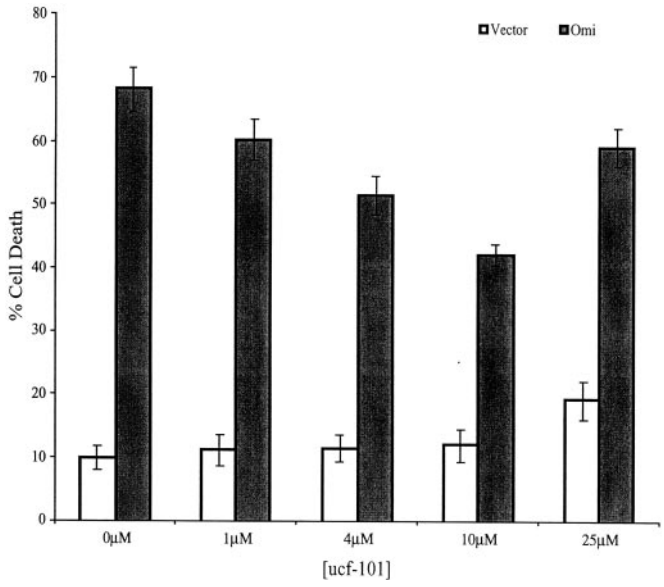

FIG. 6. **Inhibition of Omi-induced caspase independent cell death by ucf-101.** Caspase-9 (−/−) fibroblasts were transfected with pEGFP or M-Omi-EGFP (7) and kept in media containing different concentrations of ucf-101. The percentage of apoptotic cells in the transfected population was determined after 36 h as described (7).

increase for the remaining 10 min of the assay. Various concentrations of substrate, Dcasein-FITC, as well as MBP-Omi-(134–458) were used for kinetic studies.

Combinatorial Library Screening—By using an *in vitro* high throughput assay system, we screened a combinatorial library of synthetic compounds (Nanosyn). This collection represented compounds that had commonly accepted pharmaceutical hit structures with possible pharmacological properties. The assay was performed in multiple 96-well plates. The inhibition activity of each compound was expressed as the percentage of relative fluorescence change (decrease) compared with the control (no compound). The final concentration of each compound tested was 10 μM. Fifty two compounds showed more than a 20% inhibition of MBP-Omi-(134–458) activity in the initial screening, but only one compound (ucf-101) showed greater than a 50% inhibition at the concentration tested after two rounds of screening and selection (Table I).

Inhibition of the Proteolytic Activity of MBP-Omi-(134–458) Using ucf-101 and Various Analogs—We obtained several analogs of ucf-101, and the chemical structures are shown in Fig. 1. The activity of these ucf analogs was tested using MBP-Omi-(134–458) in the same *in vitro* assay system. All three analogs inhibited the proteolytic activity of MBP-Omi-(134–458) but to a lesser extent than ucf-101 (Fig. 2). ucf-104 (20 μM) inhibited 58% MBP-Omi-(134–458) activity, whereas the same concentration of ucf-101 inhibited more than 78% MBP-Omi-(134–458) activity. When MBP-Omi-(134–458) was preincubated with various concentrations of ucf-101, -102, -103, and -104 at 37 °C for longer periods (40 min or 1 h) inhibition was reduced, indicating the inhibitors did not irreversibly bind the enzyme (results not shown). MBP-Omi-(134–458) was also incubated with various amounts of Dcasein-FITC in the presence of 10 μM ucf-101. The rate of the reaction decreased by 50% when the concentration of the substrate was doubled and even reached the rate of the control reaction at higher concentrations of Dcasein-FITC (results not shown). These results suggest ucf-101 is a competitive inhibitor of Omi protease.

Assay of His-Omi-(134–458) Activity Using Unlabeled β-Casein and SDS-PAGE Analysis—The inhibitory effect of ucf-101 on the activity of His-Omi-(134–458) was monitored by incubating inhibitor and enzyme together for 10 min at room temperature prior to the addition of β-casein as a generic substrate. Fig. 3 shows that bacterially made His-Omi-(134–458) had substantial activity against β-casein, and after 30 min total degradation of the substrate occurred. ucf-101 inhibited His-Omi-(134–458) activity in a concentration-dependent manner (*lanes 4–9*) when assayed for 30 min with 200 ng of His-Omi-(134–458) and 5 μg of β-casein. ucf-101 (80 μM) was able to inhibit completely the activity of 200 ng of His-Omi-(134–458) (*lane 5*).

Comparison of the Proteolytic Activities of His-Omi-(134–458) and His-L56-(156–480) Proteins—Omi/HtrA2 and L56/

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HtrA1 are members of the same family of mammalian serine proteases with extensive homology in their catalytic domains (1). We used bacterially made His-Omi-(134–458) and His-L56-(140–480) to investigate whether ucf-101 was able to inhibit the activity of His-L56-(140–480) in a similar manner. Fig. 4 shows ucf-101 is an effective inhibitor of the activity of His-L56-(140–480) but to a lesser extent than His-Omi-(134–458).

IC_{50} of ucf-101 against Various Serine Proteases—The enzymatic activity of His-Omi and the determination of the IC_{50} for ucf-101 and ucf-102 were performed as described under "Experimental Procedures." These values are shown in Table II. ucf-101 had an IC_{50} of 9.5 μM, and ucf-102 had an IC_{50} of 45.9 μM.

The specificity of ucf-101 was determined using several unrelated serine proteases. Their susceptibility to inhibition by ucf-101 was tested. The IC_{50} values for ucf-101 inhibitor from these experiments are shown in Table III. These results suggest ucf-101 has very high specificity for the Omi protease.

Intracellular Localization of ucf-101—To investigate the potential use of ucf-101 inhibitor in *in vivo* experiments, we tested its ability to enter mammalian cells. ucf-101 has natural fluorescence at 543 nm that was used to detect the presence of the compound. HeLa cells were treated with different concentrations of ucf-101 and observed by confocal microscopy. Intense red fluorescence, due to the presence of ucf-101, was observed in the cytoplasm of the treated cells (Fig. 5)

ucf-101 Can Inhibit Omi-induced Caspase-independent Apoptosis—Mouse embryo caspase-9 (−/−) null fibroblasts (17) were transiently transfected with pEGFP-N1 (control) or M-Omi-GFP (7). Cytoplasmic expression of M-Omi-GFP induced apoptosis in ~70% of the transfected cells. When cells were treated with increasing concentrations of ucf-101, it was found that increasing the concentration of inhibitor up to 10 μM gradually reduced Omi-induced apoptosis up to 40%. ucf-101 had no effect on the control (vector alone) transfected cells. When the concentration of the ucf-101 increased to 25 μM, the anti-apoptotic activity of the inhibitor was compromised due to a cytotoxic side effect that caused apoptosis both in the M-Omi-GFP, as well as control transfected cells (Fig. 6).

DISCUSSION

Omi/HtrA2 is a mitochondrial serine protease that is released to the cytoplasm upon induction of apoptosis. In the cytoplasm, Omi binds to XIAP and relieves its inhibition of caspase-9 (6, 7, 9). In this respect, Omi acts in a manner similar to Smac/DIABLO, another mitochondrial protein that also binds to XIAP (11, 12). This interaction with XIAP is mediated via an AVPS motif that is exposed at the amino terminus of mature Omi protein after processing (6, 7). Omi is also able to induce apoptosis in a caspase-independent manner that exclusively relies on its ability to function as a protease (6–10). This caspase-independent pathway is not well understood; its contribution to overall cell death is not known, and the role of Omi as a protease in this pathway is not clear. Furthermore, although the catalytic domain of all HtrAs including Omi protein is conserved from *E. coli* to humans, the AVPS tetrapeptide is replaced at least in bovine Omi by SVLG (18). This suggests the IAP binding activity of human HtrA2 that leads to caspase-dependent apoptosis may not be critical to the overall function of Omi. Because the caspase-independent pathway relies on the ability of Omi to function as a protease, it suggests that proteolytic cleavage of specific proteins is involved. This cleavage might inactivate and remove apoptotic inhibitors, or it may activate precursor proteins whose function might be necessary for caspase-independent cell death. In order to investigate the contribution of the proteolytic activity of Omi to its overall pro-apoptotic function, we decided to screen for specific inhibitor(s) of its activity. One such specific inhibitor was identified and called ucf-101. This heterocyclic compound was able to inhibit the proteolytic activity of Omi *in vitro* in a very specific and reversible manner. The specificity of ucf-101 was tested against a panel of several unrelated serine proteases, and no significant activity was detected. When L56/HtrA1 was used in the same assay, ucf-101 showed specific inhibition against this protease. L56/HtrA1 belongs to the same family of proteases as Omi, and they both share extensive homology throughout their respective catalytic domains (1). The normal function of L56/HtrA2 is not known, and unlike Omi that localizes to mitochondria, L56 is secreted (19). The natural fluorescence of ucf-101 was used to monitor its ability to enter mammalian cells. This property is essential for ucf-101 to be useful for *in vivo* experiments designed to test inhibition of Omi that is intracellular. To test the ability of ucf-101 to inhibit the proteolytic activity of Omi *in vivo*, we used an assay where transient overexpression of a cytoplasmic form of Omi induces caspase-independent cell death (7). The cells used in this assay were caspase-9 (−/−) null mouse embryonic fibroblasts (17) in which overexpression of a cytoplasmic form of Omi can induce the caspase-independent pathway of apoptosis. This form of cell death caused by Omi relies entirely on its ability to function as a protease. Therefore, if ucf-101 blocks the activity of Omi, it will interfere with apoptosis of the caspase-9 (−/−) mouse embryonic fibroblasts. When these fibroblasts were transfected with the vector encoding the cytoplasmic Omi and treated with ucf-101, apoptosis was dramatically reduced. ucf-101, like many known protease inhibitors, was cytotoxic to mammalian cells when used at high concentrations. Therefore, the ability of ucf-101 to inhibit caspase-independent apoptosis at low doses was compromised by its cytotoxicity at higher doses. This toxicity was seen only at high concentrations of the chemical and does not exclude its use in *in vivo* experiments where it exhibits activity against Omi at much lower concentrations. ucf-101 will be a useful tool to elucidate the role of Omi in caspase-independent cell death. Several clinical conditions, such as neurodegenerative diseases, show excessive caspase-independent cell death (20, 21). If Omi through its proteolytic activity plays a role in the pathogenesis of any of these clinical conditions, ucf-101 or a similar compound would be useful for therapeutic intervention against untimely or excessive cell death.

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