A Caspase Active Site Probe Reveals High Fractional Inhibition Needed to Block DNA Fragmentation*

Received for publication, January 9, 2004, and in revised form, March 15, 2004
Published, JBC Papers in Press, April 5, 2004, DOI 10.1074/jbc.M400247200

Nathalie Méthot†, John P. Vaillancourt, JingQi Huang, John Colucci, Yongxin Han, Stéphane Menard, Robert Zamboni, Sylvie Toulmond, Donald W. Nicholson, and Sophie Roy

From the Merck Frosst Centre for Therapeutic Research, Merck Research Laboratories, Montréal, Québec H9H 3L1, Canada

Apoptotic markers consist of either caspase substrate cleavage products or phenotypic changes that manifest themselves as a consequence of caspase-mediated substrate cleavage. We have shown recently that pharmacological inhibitors of caspase activity prevent the appearance of two such apoptotic manifestations, all-spectrin cleavage and DNA fragmentation, but that blockade of the latter required a significantly higher concentration of inhibitor. We investigated this phenomenon through the use of a novel radiolabeled caspase inhibitor, [\(^{125}\text{I}\)]M808, which acts as a caspase active site probe. [\(^{125}\text{I}\)]M808 bound to active caspases irreversibly and with high sensitivity in apoptotic cell extracts, in tissue extracts from several commonly used animal models of cellular injury, and in living cells. Moreover, [\(^{125}\text{I}\)]M808 detected active caspases in septice mice when injected intravenously. Using this caspase probe, an active site occupancy assay was developed and used to measure the fractional inhibition required to block apoptosis-induced DNA fragmentation. In thymocytes, occupancy of up to 40% of caspase active sites had no effect on DNA fragmentation, whereas inhibition of half of the DNA cleaving activity required between 65 and 75% of active site occupancy. These results suggest that a high and persistent fractional inhibition will be required for successful caspase inhibition-based therapies.

The deliberate removal of excess or damaged cells is a universal feature of multicellular organisms and proceeds by a biochemical suicide program known as apoptosis (reviewed in Refs. 1 and 2). Signals that activate the apoptotic cascade include DNA damage, anoxia, lack of survival factors, and cytotoxic drugs. The downstream executioners of the apoptotic death pathway belong to a family of cysteine proteases termed caspases. Caspases exist as pro-enzymes that are activated by proximity-induced conformational change and autoprocessing, or by proteolytic processing by either an upstream activator caspase or the serine protease granzyme B (3–6). The resulting heterotetramer consists of two large and two small subunits and cleaves a limited set of protein substrates with the loosely conserved recognition sequence X-Glu-X-Asp. Cleavage occurs exclusively after the aspartic acid residue (7, 8). Seven of the 12 currently identified caspases participate in apoptosis, whereas the others play a role in inflammation (reviewed by Refs. 2 and 8).

Caspase-3 is the major effector apoptotic caspase in many cell types (9) and proteolysis of its substrates contributes to the biochemical and morphological changes that define apoptosis (10). For example, caspase-mediated cleavage of all-spectrin is believed to contribute to membrane blebbing (11, 12), whereas cleavage of ICAD leads to the internucleosomal fragmentation of DNA that typifies the apoptotic phenotype (13–15). Because of the lethal consequences of unbridled caspase activity, several checkpoints both upstream and downstream of caspase activation ensure tight control over the apoptotic pathway (reviewed in Refs. 8 and 16). Under several circumstances, however, normal safeguards do not suffice and improper caspase activation may occur. Ischemic diseases (stroke, myocardial infarction, renal ischemia, and organ transplantation), alcohol-induced hepatitis, sepsis, and Alzheimer’s and Huntington’s diseases, are conditions with demonstrated caspase-3 activation and where excessive caspase activity may contribute to the pathology (reviewed in Refs. 17 and 18). Caspase inhibitors, either polyspecific or caspase-3-specific, have proven efficacious in animal models of brain, liver, and heart ischemia (19–24), in animal models of sepsis (25), liver injury (26), and cell transplantation (27, 28). Hence, major efforts are currently underway to develop caspase inhibitors for clinical use.

Although the end point for compound efficacy is whether or not it will improve symptoms in preclinical animals models and in patients, an important aspect to drug development is to assess the percentage of target occupied by the drug, and to correlate this value with the occupancy required for effective treatment. In vivo occupancy studies for cell surface receptors have been mostly applied to anti-psychotic drugs and are performed by positron emission tomography and single photon emission-computed tomography (reviewed in Refs. 29 and 30). For intracellular enzymes, no reports of whole cell active site occupancy by inhibitors have been published. Instead, surrogate markers of enzymatic activity are used to evaluate the potency of inhibitors. For caspases, direct and indirect markers, such as substrate cleavage, phosphatidylserine exposure, and DNA fragmentation have been used to follow apoptosis in vivo and in vitro. Recently, however, we have shown that not all markers give the same indication of caspase activity (31). We speculated that the inhibition of some apoptotic manifestations require a greater percentage of caspase blockade than other markers. Here we characterized a radiolabeled, irreversible caspase inhibitor and established a methodology that enables the quantitation of active caspases in cells and the occupancy of their active sites by reversible, competitive caspase inhibitors.
We compared caspase active site occupancy with DNA fragmentation, and demonstrate that inhibition of up to 40% of caspase activity has no effect on apoptosis-induced DNA fragmentation, suggesting that a high fractional inhibition of caspases is required for inhibition of DNA cleavage. The caspase active site probe was able to detect active caspases in vivo, however, its uneven distribution precluded determination of its expression with the site occupancy method. Therefore, we devised an ex vivo method that estimates the percentage of caspase that must be blocked to inhibit DNA cleavage in vivo, and applied the methodology to the rat cecal ligation and puncture (CLP) model.

EXPERIMENTAL PROCEDURES

Caspase Active Site Probe—[125I]M808 was synthesized by the Medical Chemistry Department at Merck Frosst with a specific activity of 2000 Ci/mmol. Briefly, the synthesis was performed by peptide coupling of commercially available (5-iodo-2-methoxyphenyl)acetic acid with L-valine by addition of campothecin (3-caspase-activated DNase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid).

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of

In vivo labeling of active caspases was performed by intravenous injection (jugular vein) of 125 μl of a 0.43 μCi solution of [125I]M808 (1125 Ci/mmol) in 10% PEG200. Assuming an average blood volume of 1.25 ml per mouse, the expected initial blood concentration of
Fig. 1. Structure of the fluoromethylketone active site caspase probe [125I]M808. Characteristics of other compounds used in this work (M791, M826, and M867) are found in Refs. 22, 25, and 31.

[125I]M808 is 43 mm. Animals were euthanized 45 min after the [125I]M808 injection. Thymi were recovered and processed for protein extraction as described above. Denatured protein extracts in 1× Laemml buffer were resolved through an 18% polyacrylamide SDS gel (Invitrogen) at 30 mA in Tris glycine buffer. All samples labeled with [125I]M808 were migrated until the dye front reached 0.5 cm above the end of the gel. All material 1 cm above the dye front was cut out to remove free [125I]M808. The gels were fixed for 45 min in a 40% methanol, 10% acetic acid solution and dried for 90 min at 80 °C under vacuum before exposure on Kodak film with MS intensifying screens (Kodak).

Western Blotting—Proteins were resolved in an 18% SDS-polyacrylamide gel and transferred onto a 45-μm nitrocellulose membrane (Invitrogen) in Tris glycine, 20% methanol buffer at 40 V for 2 h. Non-specific protein binding was minimized by incubating the membranes for 1 h in blocking buffer (5% nonfat milk, TBS, 0.1% Tween 20). Caspase-3 antisera MF R280 and MF467 were raised in rabbits against recombinant large subunits (p17) of human and rat caspase-3, respectively. Both antisera were used at a 2000-fold dilution in blocking buffer and incubated for 1 h at 25 °C. Membranes were washed in TBS, 0.1% Tween 20 and incubated for 45 min with horseradish peroxidase-coupled anti-rabbit IgG antibody (Amersham Biosciences) diluted 5000-fold in blocking buffer. The enhanced chemiluminescence reaction was performed with Supersignal West Femto chemiluminescent reagent (Pierce) and exposed to Hyperfilm ECL (Amersham Biosciences). Densitometry on 125I-exposed or chemiluminescence-exposed film was obtained using a Bio-Rad GS800 instrument and QuantityOne software (Bio-Rad). A pixel saturation calibration curve was done before each scan, with saturated pixels appearing in red. Only signals that were well below the apparatus pixel saturation limit were quantitated. Background densitometric values corresponding to the identical area size of each quantitated bands were subtracted. In some experiments, an [125I]M808-labeled caspase-3 standard curve was performed to establish the linearity of the signal. A 20-fold dynamic range with a single film exposure time was achieved.

**IC50 Calculations and Statistical Analysis**—Curve fitting was obtained using a sigmoidal Hill 4-parameter equation using SigmaPlot 8.0 software. Statistical analysis was performed by standard one-way analysis of variance. Where applicable, data were log-scaled so that underlying assumptions of equal variance and normality were better satisfied. All comparisons were deemed statistically significant at the 0.05 level.

**RESULTS**

**[125I]M808 Specificity and Sensitivity in Vitro—**[125I]M808 is an iodinated trifluoromethylketone caspase inhibitor predicted to bind covalently and irreversibly to the active site cysteine located in the large caspase subunit (Fig. 1). The inhibitory profile of M808 against purified recombinant caspases indicates a slight preference (2-fold) for caspase-3 over caspase-8, and a 10-fold preference over caspase-7 (Table I).

We tested the specificity of [125I]M808 for active caspases in crude protein extracts. Equal amounts rat tissue homogenates were either incubated with [125I]M808 or pretreated with granzyme-B to activate caspases prior to the addition of [125I]M808. In parallel, caspase activity in granzyme-B-treated extracts was measured by cleavage of the fluorogenic substrate Ac-DEVD-AMC. The large subunit of fully processed caspase-3 migrated at 17 kDa (p17). Partially processed caspase-3 retains part (p19) or all (p20) of the pro-domain (34), but is as active as the p17 form. Removal of the prodomain is self-catalyzed (33). As expected, the p17 subunit of purified recombinant human caspase-3 covalently bound [125I]M808 (Fig. 2A, first lane). All granzyme-B-treated rat tissues contained an [125I]M808-labeled 19-kDa protein, whereas liver and thymus showed an additional polypeptide migrating at 20 kDa (second through fifth lanes). The identity of the radiolabeled proteins as caspases was ascertained by several means. Radiolabeled proteins migrating at this position were not observed when granzyme-B was omitted (data not shown). Moreover, preincubation of the extract with the caspase-3-specific inhibitor M791 abolished (sixth through eighth lanes) or greatly reduced (ninth lane) the appearance of the radiolabeled proteins. Caspase activity, as measured by Ac-DEVD-AMC cleavage, correlated with the amount of radiolabeled p19/p20 proteins (Fig. 2B). [125I]M808 recognized polypeptides migrating at 17, 19, and 20 kDa in extracts of cells treated with the apoptotic inducers camptothecin or anti-Fas antibody. Labeling of a 70-kDa protein was also observed in NT2 cell extracts, but not Jurkat (Fig. 2C). The p17-, p19-, and p20-radiolabeled proteins were absent if the apoptotic inducer was omitted, or if M791 had been added in the extract, whereas labeling of the 70-kDa polypeptide was not affected. Taken together, these results demonstrate that [125I]M808 binds to caspases, including caspase-3, in a complex protein mixture.

**Table I.** M808 affinity for various caspases

| Caspase | K<sub>IC50</sub> (mM) |
|---------|------------------|
| Caspase-1 | 75.733 |
| Caspase-2 | 37.550 |
| Caspase-7 | 2.107 |
| Caspase-8 | 12.850 |

**a** S. Roy, unpublished data.
types of ischemia-injured tissues and in the thymus from septic animals.

$[^{125}I]M808$ Measures Caspase Activity in Vitro—We have shown that $[^{125}I]M808$ is a sensitive probe that detects active caspases in apoptotic cells or in injured tissues. Next, we examined whether $[^{125}I]M808$ could be used to measure the fraction of active caspase-3 remaining in the presence of a reversible site inhibitor. Purified recombinant caspase-3 was preincubated with increasing amounts of the reversible, competitive, and caspase-3-selective inhibitors, M791 or M826 (22, 25). Half of the reaction was used for the Ac-DEVD-AMC cleavage rate assay, whereas the other half was incubated with $[^{125}I]M808$ before resolving on SDS-PAGE. The $K_i$ value for M791 measured by Ac-DEVD-AMC cleavage was 0.74 nM (Fig. 3A). Remarkably, the $K_i$ value for M791, when enzyme occupancy was measured with $[^{125}I]M808$, was virtually identical at 0.73 nM (Fig. 3, C and E). Similarly, the $K_i$ values for M826, measured by Ac-DEVD-AMC and $[^{125}I]M808$ labeling, were
Incubation with \([^{125}\text{I}]\text{M808}\) for 60 min instead of 5 increased by 2-fold the apparent \(K_i\) value for M791 (data not shown), most likely because of the dissociation of M791 and its replacement by \([^{125}\text{I}]\text{M808}\). Thus, the caspase active site probe \([^{125}\text{I}]\text{M808}\) can be used to quantify the amount of free active caspase-3 in a mixture of enzyme and reversible inhibitor.

**[125I]M808 Measures Caspase Active Site Occupancy in Cells**—We then assessed whether \([^{125}\text{I}]\text{M808}\) could measure caspase active site occupancy by reversible inhibitors in living cells. Jurkat cells were treated with camptothecin for 5 h to induce apoptosis, in the presence or absence of M791. \([^{125}\text{I}]\text{M808}\) was added to the culture media for the last incubation hour. M791, which is a reversible and membrane-permeable caspase-3-selective inhibitor, blocked cell apoptosis as measured by DNA fragmentation with an \(IC_{50}\) of 4.8 \(\mu\text{M}\) (Fig. 4A). Western blot analysis of cell lysates using a caspase-3 antibody showed that M791 prevented the autoprocessing of p20 caspase-3 into p19 and p17, but did not block the overall formation of p20, as expected for a caspase-3 selective inhibitor (Fig. 4B). \([^{125}\text{I}]\text{M808}\) labeled all three forms of caspase-3 (Fig. 4C). Typically, 30% of the total pool of active caspases were

---

**Fig. 3. Active site occupancy by caspase inhibitors, measured by \([^{125}\text{I}]\text{M808}\) labeling, correlates with inhibition of Ac-DEVD-AMC cleavage.** A and B, caspase inhibitor titration and Ac-DEVD-AMC cleavage activity with recombinant human caspase-3. A, M791, B, M826. Both of these inhibitors are competitive and reversible. Under the fluorogenic substrate assay conditions, \(K_i = IC_{50}/2\). The \(K_i\) and IC\(_{50}\) values obtained are indicated on the graphs. C and D, \([^{125}\text{I}]\text{M808}\) labeling of purified recombinant human caspase-3 preincubated 15 min with either (C) M791 or (D) M826. Me\(_2\)SO was used as diluent to a final concentration of 1%. The arrows point to the radiolabeled caspase-3 p17 subunit. The \(K_i\) values for M791 and M826 were obtained by sigmoidal curve fitting of densitometric measurements for \([^{125}\text{I}]\text{M808}\)-labeled p17 and correspond to the inflection point. Here, \(K_i = IC_{50}\) when the substrate concentration is zero. The concentration of inhibitor present is indicated below each lane. E and F, densitometric measurement of \([^{125}\text{I}]\text{M808}\)-labeled p17 shown in panels C and D. The densitometric value of the Me\(_2\)SO control for M791 served as control for M826.
labeled under the conditions used (data not shown). The occupancy of active caspase-3 by M791 was evident from the decrease in the sum of radiolabeled p17/p19/p20 intensity as the concentration of M791 increased (Fig. 4C). A concentration of 2.1 μM M791 was required to block 50% of the caspase active sites, whereas 50% inhibition of DNA fragmentation necessitated 4.8 μM (Fig. 4, A and D). This suggests that a large fractional inhibition of caspase-3 is required to inhibit apoptosis.

Apoptosis and caspase-3 activation has been documented in thymocytes and splenocytes of septic rats and mice (31, 37, 38). Caspase-3 selective inhibitors such as M791 and M867, together with the absolute requirement for ICAD cleavage to allow DNA fragmentation (39), suggest that thymocyte apoptosis in vitro and during sepsis is mediated by caspase-3, and possibly caspase-7. M867 completely blocks apoptosis in vivo in thymi of septic rats, but with strikingly different efficacy, depending upon whether αII-spectrin cleavage or DNA fragmentation was used to assess apoptosis (31). Similar results were also obtained with cultured rat thymocytes, where 3-fold more M867 was required to inhibit 50% of the DNA fragmentation than αII-spectrin cleavage (31). In light of these results, we compared the potency of M867 at inhibiting DNA fragmentation with caspase enzyme occupancy, in cultured rat thymocytes. Cleaved caspase-3 and DNA fragmentation were quantified in parallel by Western blotting and flow cytometric determination of subdiploid cells. Shown in Fig. 5 is a representative example of five independent experiments. Western blot showed a minimal impact of M867 on caspase-3 cleavage except at the highest inhibitor concentration, as expected for a caspase-3 selective compound, given that its activation is presumed to be mediated by upstream caspases (Fig. 5A). M867 inhibited DNA fragmentation in cultured rat thymocytes with an IC50 of 0.24 μM (Fig. 5C). Incubation of thymocytes for 15 min with [125I]M808 labeled p17, p19, and p20 caspase subunits in healthy and apoptotic Jurkat cells exposed to increasing amounts of M791. The p17, p19, and p20 radiolabeled polypeptides are indicated by arrows. No labeling of full-length, unprocessed p32 caspase was observed. D, plot of [125I]M808-labeled p17/p19/p20 densitometry against M791 concentration. The IC50 determined by caspase active site occupancy is 2.1 μM.

Fig. 4. Inhibition of DNA fragmentation in apoptotic Jurkat cells requires greater than 50% caspase occupancy. A, percentage of residual DNA fragmentation activity and corresponding IC50 value in apoptotic Jurkat cells treated with increasing amounts of M791. DNA fragmentation here and in all subsequent figures was measured by flow cytometry and was equated to the percentage of subdiploid cells. 100% DNA fragmentation activity was arbitrarily set at the percentage of subdiploid cells present in the absence of caspase inhibitor. B, caspase-3 Western blot on camptothecin-treated Jurkat cell extracts. Unprocessed (p32), fully (p17) and partially processed (p19 and p20) caspase-3 polypeptides are indicated by arrows. The concentration of M791 present is shown below each lane. C, [125I]M808-labeled p17, p19, and p20 caspase subunits in healthy and apoptotic Jurkat cells exposed to increasing amounts of M791. The p17, p19, and p20 radiolabeled polypeptides are indicated by arrows. No labeling of full-length, unprocessed p32 caspase was observed. D, plot of [125I]M808-labeled p17/p19/p20 densitometry against M791 concentration. The IC50 determined by caspase active site occupancy is 2.1 μM.
concentration, only 15% of DNA fragmentation was inhibited (Fig. 5C). The average IC₅₀ for M867 determined by DNA fragmentation in five independent experiments was 0.27/0.04 M. The same experiments yielded an average IC₅₀ of 0.13/0.02 M when caspase activity was measured with [125I]M808. The apparent 2-fold decrease in M867 potency with respect to DNA fragmentation is statistically significant (p = 0.003). In contrast, the concentration of M867 required to reduce II-spectrin cleavage by half was not statistically different from the dose needed to achieve 50% occupancy of caspase active sites (data not shown). Other compounds tested in Jurkat cells and rat thymocytes showed that a 50% inhibition of DNA fragmentation required between 62 and 77% of caspase active sites to be blocked (Table III).

**TABLE III**

| Cell line | Inhibitor | IC₅₀ DNA cleavage | Occupancy at IC₅₀ |
|-----------|-----------|-------------------|------------------|
| Jurkat    | M791      | 4.8               | 77               |
| Thymocytes| M791      | 4.8               | 65               |
| Thymocytes| M826      | 0.14              | 62               |
| Thymocytes| M867      | 0.27              | 70               |

Not in sham animals (Fig. 6C). Thus, [125I]M808 detected active caspases in vivo. However, in contrast to protein extracts and whole cell labeling, the intensity of the p17 signal was not proportional to the amount of p17 caspase-3 present. This was also true for thymus proteins non-specifically labeled with [125I]M808. This discrepancy may be due to the variable accessibility of the probe to the injured thymus during sepsis, as this condition is known to cause organ hypoperfusion. As such, even though [125I]M808 detected active caspases in vivo, its usefulness is limited to systems where tissues are equally perfused in all animals.

**Fractional Inhibition of Caspases in an ex Vivo Assay**—Because in vivo injection of [125I]M808 did not allow caspase
fractional inhibition determination, we tested whether active site occupancy could be obtained by labeling cells *ex vivo*. Rats were subjected to CLP surgery and infused continuously with either vehicle or M867 at 1 or 4 mg/kg/h. The doses of M867 chosen were expected to have either no effect or partial inhibitory effects on thymocyte DNA fragmentation (31). Thymi were collected 24 h post-surgery and single cell suspensions were prepared for DNA fragmentation analysis and whole cell *ex vivo* [125I]M808 labeling. Relative caspase-3 amounts were also quantified by Western blotting. As expected, CLP surgery resulted in a significant increase in the caspase-3 p17 fragment content of thymocytes. Infusion of M867 at either dose did not significantly affect p17 levels (Fig. 7A). M867 at 4 mg/kg/h reduced DNA fragmentation by 70%, whereas 1 mg/kg/h failed to inhibit (Fig. 7C). The slight increase in DNA cleavage observed by treatment with 1 mg/kg/h while statistically significant here, was not observed in other experiments (31). Active caspases were labeled in suspended thymocytes obtained from all treatment groups (Fig. 7B). There was a positive correlation between the amount of caspase-3 p17 fragment as measured by Western blotting, and the amount of [125I]M808 p17 polypeptide (*r* = 0.92; Fig. 7D). This positive correlation was used to calculate the percentage of caspase active sites occupied by M867, much in the same manner as what was outlined in Table II. A mean fractional inhibition of 23 and 62% was found in the 1 and 4 mg/kg/h groups, respectively (Fig. 7E). Thus, caspase fractional inhibition measured in an animal model of cellular injury correlates well with the amount of M867 dosed and the percentage of DNA fragmentation inhibition achieved.

**DISCUSSION**

We describe here a novel assay that enables the determination of fractional inhibition of an intracellular enzyme, caspase-3, by a reversible active site inhibitor. We have shown the caspase probe [125I]M808 to be a highly sensitive tool capable of detecting active caspases in several animal models of apoptotic injury. No labeling of unprocessed, full-length caspases was observed with this probe. Partial labeling of the total pool of free caspases with [125I]M808 accurately reflects the amount of caspase activity remaining, both with purified enzyme and in living cells. The fact that fractional inhibition determination was feasible on a short-lived enzyme such as caspase-3 suggests that this method will also be applicable to other enzymes. Using this method, we show that high fractional inhibition of caspases is required to block DNA fragmentation in cultured cells.

The determination of caspase occupancy described in this work makes use of intact cells as opposed to lysed cells or
homogenized tissues that had been exposed to M867. In theory, it should have been possible to measure fractional inhibition by M867 with direct exposure of lysates to \[^{125}\text{I} \text{M808}\]. However, we found that cells or tissues incubated with M867 or M826, and subsequently lysed and exposed briefly to \[^{125}\text{I} \text{M808}\], exhibited far greater enzyme occupancy relative to the IC\text{50} determined by DNA fragmentation (data not shown). We speculate that much of M867 and M826 are membrane-associated in live cells and freed upon lysis, thus gaining access to free caspase active sites. This is a compound-specific effect because caspase active site occupancy by M791 is the same whether determined on living or lysed cells.3

Although the \[^{125}\text{I} \text{M808}\] caspase active site probe has proven itself extremely useful for \textit{ex vivo} fractional inhibition determination, the methodology suffers from a few limitations. Occupancy of caspase active sites by a reversible inhibitor must be determined rapidly to minimize dissociation of the reversible inhibitor, and will be artificially low if the drug has a short dissociation half-life. For M826 and M867, the dissociation half-life with purified enzyme is 60 min at 25°C (data not shown). We speculate that much of M867 and M826 are membrane-associated in live cells and freed upon lysis, thus gaining access to free caspase active sites. This is a compound-specific effect because caspase active site occupancy by M791 is the same whether determined on living or lysed cells.3

One of the major obstacles to fractional inhibition determination with \[^{125}\text{I} \text{M808}\] is its limitation to systems where the large subunit of cleaved caspases can be detected by Western blotting. Additionally, if an inhibitor is prone to membrane association, such as is likely the case with M867, occupancy must be determined in tissues where intact cells can be rapidly isolated. In this respect, CLP-induced sepsis is a good model to study \textit{in vivo} fractional inhibition because large amounts of p17 are generated in the thymus, and thymocyte cell suspensions can be rapidly made. The \textit{ex vivo} caspase active site occupancy determined in septic animals dosed with M867 is probably a slight underestimation of the actual value, because of M867 dissociation during tissue processing time. A more accurate determination of fractional inhibition might be achievable by directly labeling the tissue extract, as opposed to whole cells. A

---

3 N. Méthot, unpublished results.
caspase inhibitor that does not accumulate in membranes, such as M791, would need to be used to compare occupancy measured by ex vivo labeling, and occupancy measured by direct labeling of tissue extracts. Unfortunately, M791 was not sufficiently efficacious in rat septicemia to test this hypothesis.

The [125I]M808 caspase active site probe also has potential utility in clinical caspase inhibitor development for sepsis. Although apoptotic cells are known to be rapidly cleared from the circulation, apoptotic peripheral blood monocytes and lymphocytes have been detected in septic patients or in patients undergoing chemotherapy (42–44). It would be interesting to test whether [125I]M808 can detect active caspases in whole blood, and eventually, as caspase inhibitors reach clinical trials, determine occupancy ex vivo in blood of patients.

In summary, we have measured caspase active site occupancy by potent, reversible inhibitors using a radiolabeled active site probe. Our data indicates that a fractional inhibition between 65 and 75% is required to block DNA fragmentation by 50%, and explains in part why a 3–4-fold higher concentration of M867 is needed to oppose DNA cleavage compared with o1-spectrin cleavage. As prevention of DNA fragmentation is most likely sine qua non for cell survival, our findings suggest that a high and persistent blockage of caspase active site will be needed for therapeutic benefit.

Acknowledgments—We are indebted to D. Normandin, S. Wong, C. Desroches, C. Jones, G. Castonguay, L. Belair, M. Lavoie, R. Basori, K. Ortega, S. Lévesque, J. Rozon, D. Gendron, and N. Kelly for animal care, surgical help, clinical signs monitoring, and necropsies. We thank S. Toumlond for middle cerebral artery occlusion cortex and hypoxia-ischemia brains, and N. Thornberry for purified recombinant human caspase-3. We are grateful to D. Percival for critical reading of the manuscript.

REFERENCES

1. Hengartner, M. O. (2000) Nature 407, 770–776
2. Adams, J. M. (2000) Science 289, 1321–1325
3. Martin, S. J., Amarante-Mendes, G. P., Shi, L., Chung, T. H., Casiano, C. A., O’Brien, G. A., Fitzgerald, P., Tan, E. M., Bokoch, G. M., Greenberg, A. H., and Green, D. R. (1999) EMBO J. 18, 2407–2416
4. Medema, J. P., Toes, R. E., Scaffidi, C., Zheng, T. S., Flavell, R. A., Melief, C. J., Wang, L., Lu, M., and Chopp, M. (2002) J. Biol. Chem. 277, 29300–29309
5. Buchman, T. G., and Karl, I. E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 15451–15456
6. Biggar, D., Han, Y., Nicholson, D. W., and Karl, I. E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 30128–30136
7. Curr. Pharm. Des. 6, 973–989
8. van Waarde, A. (2000) J. Biol. Chem. 275, 18411–18414
9. Black, S. C., Huang, J. Q., Rezaiefar, P., Radiovic, S., Eberhart, A., Nicholson, D. W., and Bodger, I. W. (1998) J. Mol. Cell Cardiol. 30, 733–742
10. Wang, S.-D., Huang, K.-J., Lin, Y.-S., and Lei, H.-Y. (1994) J. Immunol. 152, 5014–5021
11. Hotchkiss, R. S., Tinsley, K. W., Swanson, P. E., Tinsley, K. W., Chang, K. C., Roy, S., Black, C., Grimm, K., Aposhian, R., Han, Y., Nicholson, D. W., and Karl, I. E. (2000) J. Exp. Med. 199, 199–207
12. Reeves, B., Bristow, B., Heng, R., Leutwiler, A., Mueller, R., and Wuehrman, H.-J. (1994) Tissue Cell. 36, 929–935
13. Roy, S., Faye, C. I., Garreau, H., Karmann, S., Keen, S. L. C., Rowland, K., Seiden, I. M., Thornberry, N. A., and Nicholson, D. W. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6132–6137
14. Garcia-Calvo, M., Peterson, E. P., Rasper, D. M., Vaillancourt, J. P., Robert, C., Rodriguez, M., Nicholson, D. W., and Thornberry, N. A. (1999) Cell Death Differ. 6, 362–369
15. Schlegel, J., Peters, I., Orrenius, S., Miller, D. K., Thornberry, N. A., Yavin, E., and Curr. Pharm. Des. 6, 733–739
16. Puthalakath, H., and Strasser, A. (2002) Blood 98, 3066–3073
17. Endres, M., Namura, S., Shimizu-Sasamata, M., Waeber, C., Zhang, L., Gomez-Isla, T., Hyman, B. T., and Muckwitz, M. A. (1998) J. Cereb. Blood Flow Metab. 18, 238–247
18. Reed, J. C. (2002) Nat. Rev. Drug Discov. 1, 111–121
19. Contreras, P. C. (2001) J. Neurosci. 21, 1593–1610
20. Ayala, A., Herdon, C. D., Lehman, D. L., Ayala, C. A., and Chaudry, I. H. (1998) J. Trauma. 45, 620–626