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Selective and Sensitive Monitoring of Caspase-1 Activity by a Novel Bioluminescent Activity-Based Probe

Maik Kindermann,[1,4] Heidi Roschitzki-Voser,[2,4] Dejan Caglič,[3,4] Urška Repnik,[3] Catherine Miniejew,[1] Peer R.E. Mittl,[2] Gregor Kosec,[3] Markus G. Grütter,[2] Boris Turk,[3,*] and K. Ulrich Wendt[1,*]
[1]Sanofi-Aventis Deutschland GmbH, R&D Chemical and Analytical Sciences, Industriepark Park Hoechst, 65926 Frankfurt, Germany
[2]University of Zürich, Department of Biochemistry, Winterthurerstrasse 190, 8057 Zürich, Switzerland
[3]Jožef Stefan Institute, Department of Biochemistry, and Molecular and Structural Biology, Jamova 39, 1000 Ljubljana, Slovenia
[4]These authors contributed equally to this work
*Correspondence: boris.turk@ijs.si (B.T.), ulrich.wendt@sanofi-aventis.com (K.U.W.)
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SUMMARY

The role of caspase-1 in inflammation has been studied intensely over recent years. However, the research of caspase-1 has remained difficult mainly due to the lack of sensitive and selective tools to monitor not only its abundance but also its activity. Here we present a bioluminescent activity-based probe (ABP) for caspase-1, developed by the *Reverse Design* concept, where chemically optimized protease inhibitors are turned into selective substrate ABPs. The probe exhibits excellent selectivity for caspase-1 and ~1000-fold increase in sensitivity compared to available fluorogenic peptidic caspase-1 substrates. Moreover, we have been able to monitor and quantify specific caspase-1 activity directly in cell lysates. The activity correlated well with processing of prointerleukin-1β and prointerleukin-18 in phorbol 12-myristate 13-acetate (PMA)-stimulated cells. A detectable caspase-1 activity was present also in nonstimulated cells, consistent with processing of constitutively expressed prointerleukin-18.

INTRODUCTION

Caspase-1 (interleukin-1β converting enzyme [ICE]) belongs to the caspase family of cysteine endopeptidases (Alnemri et al., 1996) that play a key role in the initiation and execution of apoptosis and inflammation. Caspase-1 was the first caspase identified and represents the prototypic member of the family of proinflammatory caspases (Black et al., 1989; Kostura et al., 1989). The main substrate of caspase-1 is the proinflammatory cytokine interleukin-1β (IL-1β). On induction with proinflammatory stimuli, proIL-1β is expressed as inactive cytoplasmic precursor (p35) that must be proteolytically processed to generate the mature active form (p17) (Thornberry et al., 1992). Another important caspase-1 substrate, prointerleukin-18 (proIL-18), which is structurally similar to proIL-1β, is also synthesized as a biologically inactive precursor that is, however, constitutively expressed in a variety of cells (Dinarello, 1999).

Like most proteolytic enzymes, caspase-1 is synthesized as an inactive zymogen, which requires activation. Caspase-1, carrying a long N-terminal prodomain, is suggested to belong to the family of apical caspases together with related proapoptotic initiator caspases-2, -8, -9, and -10 (Baliga et al., 2004; Boatright et al., 2003; Donepudi et al., 2003; Wang et al., 2001). On the basis of the initial studies of caspase-8 and caspase-9 activation, it was suggested that they are activated by oligomerization, which is assisted by multiprotein platforms such as DISC or apoptosome and not by proteolytic cleavage within the linker region (Salvesen and Dixit, 1997). Internal proteolysis was initially believed not to activate these caspases but was rather a secondary event and resulted in partial stabilization of the active dimer (Boatright et al., 2003). However, a recent report demonstrated that neither dimerization nor cleavage of caspase-8 alone was sufficient to activate caspase-8 (Keller et al., 2010; Oberst et al., 2010). The discovery of inflammasome, a multiprotein scaffold that assists caspase-1 oligomerization, paved the way to better understanding of caspase-1 activation in cells challenged with specific microbial or danger signals (Martinon et al., 2002; Martinon and Tschopp, 2004; Srinivasula et al., 2002). Several inflammatory diseases are linked to caspase-1 activity such as septic shock, inflammatory bowel disease, familial cold auto-inflammatory syndrome, rheumatoid arthritis, osteoarthritis, and gout (Cornelis et al., 2007; Joshi et al., 2002; Martinon et al., 2006). On the other hand, caspase-1 deficiency leads to increased susceptibility for bacterial infection (Lara-Tejero et al., 2006), demonstrating the importance of caspase-1 activation for host defense (Faustin et al., 2007).

However, only a very minor portion of caspase-1 is usually found to be proteolytically processed after inflammatory stimulation (Ayala et al., 1994). Moreover, because activation of procaspase-1 is likely not critically dependent on enzyme processing but multiprotein platform-assisted oligomerization, immunodetection with antibodies or proteomics methods may likely fail to distinguish between the expression level (physical abundance) of caspase-1 and its actual proteolytic activity in vivo. This inherent limitation in protease biochemistry would be overcome

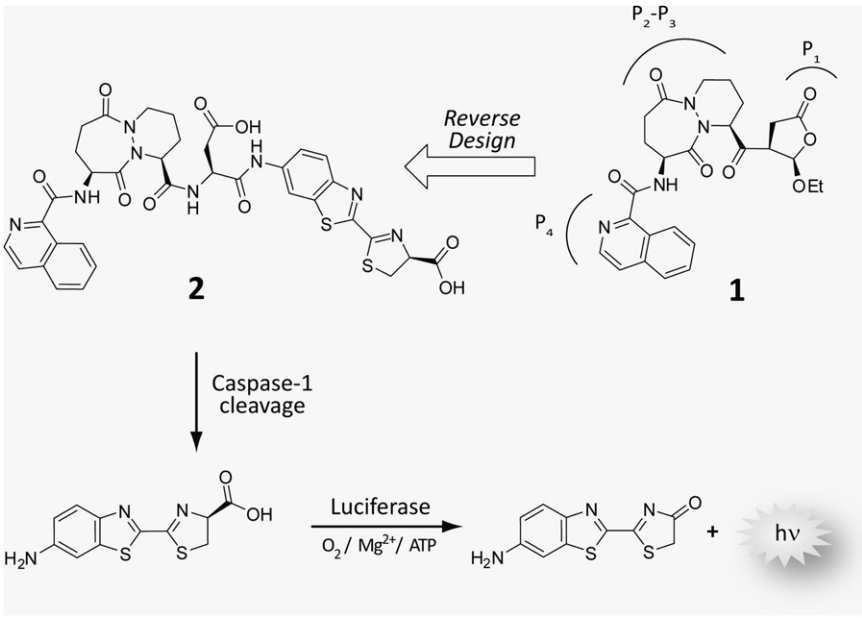

Figure 1. *Reverse Design* of CM-269
The scaffold of the bioluminescent substrate CM-269 is based on the caspase-1 inhibitor Pralnacasan 1 (molecule **1**). The concept of *Reverse Design* transferred the selectivity profile of the optimized inhibitor into the bioluminescent substrate CM-269 (molecule **2**) by replacing the warhead of the inhibitor with a cleavable peptide bond and subsequently attaching amino-luciferin as reporter group (see also Supplemental Scheme and Figure S1 for details). Enzymatic turnover of CM-269 was tested in a coupled enzymatic assay containing recombinant firefly luciferase as the reporter enzyme. Cleavage of the luciferin-coupled substrate generated free amino-luciferin which was subsequently oxidized by luciferase resulting in emission of light.

by the use of sensitive and selective activity-based probes (ABPs) enabling a direct detection of caspase-1 activity, and would contribute to the elucidation of the molecular processes in which caspase-1 has been shown to play a role, and would open venues for novel therapeutic interventions.

We recently proposed that highly selective substrate ABPs with reduced peptidic character can be obtained by a simple chemical concept, which we referred to as *Reverse Design* (Watzke et al., 2008). The concept is based on turning highly selective protease inhibitors with chemically optimized structures into selective ABPs. We successfully applied this concept to transfer the selectivity profile of two selective cathepsin inhibitors into selective ABPs by replacing the substrate-mimicking warhead of the inhibitors with a cleavable peptide bond and subsequently attaching appropriate reporter groups (Watzke et al., 2008).

In the present work, we have applied the chemical concept of *Reverse Design* to turn the highly selective caspase-1 inhibitor Pralnacasan (Figure 1, molecule **1**) (Siegmund and Zeitz, 2003) into a bioluminescent caspase-1 selective ABP, referred to as CM-269 (Figure 1, molecule **2**). CM-269 was validated using recombinant human caspases and whole-cell lysates. The probe exhibits good kinetic properties and superior selectivity toward caspase-1 and is sensitive enough to allow direct monitoring of caspase-1 activity in complex proteomic samples. We were able to detect caspase-1 activity in whole-cell lysates of stimulated and nonstimulated monocytic cells. Our results demonstrate that considerable caspase-1 activity is present already in nonstimulated immune cells, which correlates with processing of constitutively expressed proIL-18 in nonstimulated cells. Using CM-269 we were able to further show that in phorbol 12-myristate 13-acetate (PMA)-stimulated cells the activity of caspase-1 was significantly increased, which may be required for efficient processing of its natural substrate proIL-1β.

RESULTS

Synthesis and In Vitro Characterization of the Bioluminescent ABP for Caspase-1

The highly selective caspase-1 inhibitor Pralnacasan (Linton, 2005; Siegmund and Zeitz, 2003) provided an attractive scaffold for the *Reverse Design* of a bioluminescent ABP for caspase-1. The resulting bioluminescent CM-269 was synthesized by solid-phase peptide chemistry on a 2-chlorotrityl resin (see Supplemental Experimental Procedures available online). Enzymatic turnover of CM-269 was tested in a coupled enzymatic assay containing recombinant firefly luciferase as a reporter enzyme. Cleavage of the luciferin-coupled substrate generated free amino-luciferin, which was subsequently oxidized by luciferase resulting in emission of light (Figure 1). Maximum signal was detected when the steady state between the caspase-1 and luciferase reactions was achieved. Different from fluorescent measurement, where the signal of the accumulating product can be displayed as a velocity (e.g., relative fluorescence over time), the intensity of the emitted light in the steady state of a bioluminescent assay reaches a plateau. A set of control experiments was carried out to obtain optimal conditions resulting in a maximum signal of the coupled assay (Figures S2–S4). A stable signal could be obtained within 20 min for caspase-1 concentrations of 0.01 to 10 nM (Figure S3D). To determine the kinetic parameters (K_m, k_{cat}) of probe hydrolysis, the coupled assay was "uncoupled" and the two reaction steps, cleavage of the probe by caspase-1 and the subsequent oxidation of amino-luciferin by luciferase, were carried out consecutively. In this experiment, CM-269 was shown to be efficiently cleaved by caspase-1 with a k_{cat} value of 0.96 s^{-1} and a K_m value of 4.33 × 10^{-6} M.

Even though product accumulation in fluorescent assays generally results in higher intensities than for bioluminescent readouts, more sensitive signals are achieved in bioluminescence due to the very low background levels (Troy et al., 2004). To compare the sensitivity of the CM-269 bioluminescent assay with the sensitivity of fluorescent measurement using the commercially available caspase-1 substrate Ac-WEHD-AMC, both the peptidic substrate and the ABP were assayed with caspase-1 ranging from 0.005 nM to 100 nM. As shown in Figure 2,

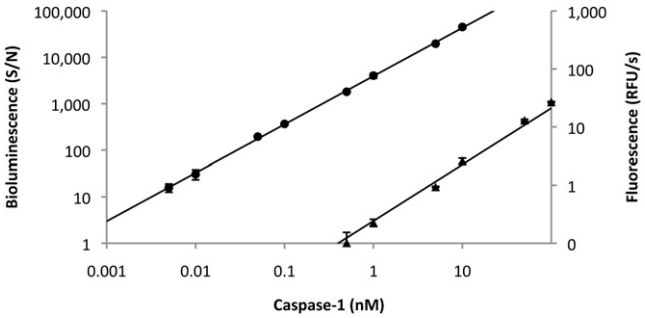

Figure 2. CM-269 Exhibits Improved Sensitivity over Standard Fluorogenic Substrate Ac-WEHD-AMC

Sensitivity of bioluminescent CM-269 (circles; left y axis) and fluorescent substrate Ac-WEHD-AMC (triangles; right y axis) were tested at substrate saturation (30 μM) with 5 pM to 100 nM active caspase-1. To verify the linearity, the results were plotted on a logarithmic scale. Each measurement was carried out in triplicate. Error bars indicate standard deviations from the mean value. See also Figure S2 and Figure S3 for optimization of coupled assay conditions.

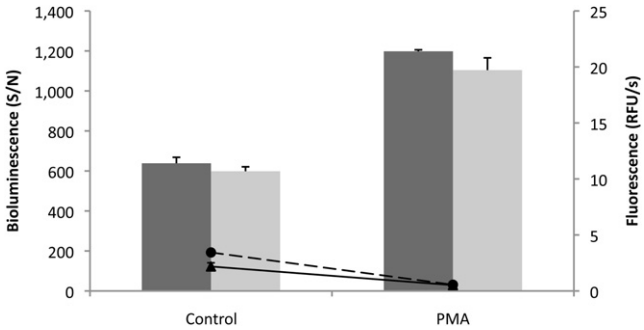

Figure 4. Caspase-1 Activity Increases in PMA-Stimulated Cells

The bioluminescent signal of CM-269 (3 μM) is shown as bars on the left (THP-1 in black and U937 in gray) and the fluorescent signal of Ac-DEVD-AFC (10 μM) as line plot (THP-1 dashed line, U937 solid line) on the right axis. In both cell lines, phorbol 12-myristate 13-acetate (PMA)-stimulation resulted in increased cleavage of CM-269, whereas no increase in Ac-DEVD-AFC cleavage was observed. At least three independent experiments were carried out. Error bars indicate standard deviations from the mean value.

the detection limit of active caspase-1 using CM-269 was at about 5 pM, whereas significantly higher enzyme concentrations (1 nM) were required for the detection of active caspase-1 when using the conventional fluorogenic substrate Ac-WEHD-AMC.

To test the in vitro selectivity of the probe, CM-269 was profiled against a panel of recombinant human caspases and recombinant human granzyme B, a proapoptotic serine protease. The substrate binding site of granzyme B is similar to that of the caspase family of proteases and in both cases substrate hydrolysis occurs after an aspartic acid (Thornberry et al., 1997). The selectivity, expressed as $(S/N)_{max}/K$, was derived from the slope of a S/N versus $[E]_t[S]$ plot (see Experimental Procedures for details). As depicted in Figure 3, the rate of substrate hydrolysis of CM-269 was highest for caspase-1, being about ten times higher than for any other caspase tested in this experiment or granzyme B. Selectivity of CM-269, although bearing a rather large moiety enabling a bioluminescent readout, was similar to the selectivity of Ac-WEHD-AMC (Figure S5), which has been shown to be cleaved only by proinflammatory caspases-1, -4, and -5 and not by the proapoptotic caspases (McStay et al., 2008; Pereira and Song, 2008; Thornberry, 1997).

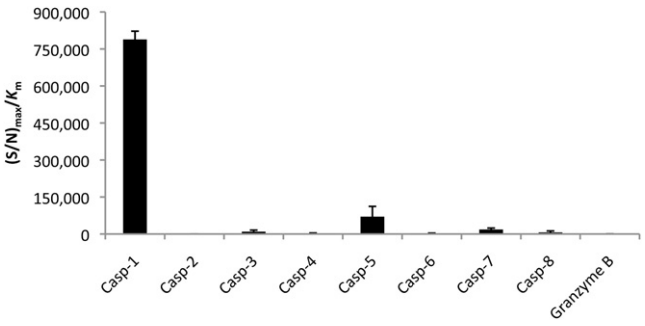

Figure 3. CM-269 Is Selective for Recombinant Caspase-1

In vitro selectivity of CM-269 was tested at K_m (4 μM) against a panel of recombinant human caspases and recombinant human granzyme B. All enzymes were applied at 10 nM active protein concentrations. $(S/N)_{max}/K_m$ values (see Equation 5) derived from the slope of the S/N versus $[E]_t[S]$ plot denoting the selectivity parameters (see Experimental Procedures for details). Each measurement was carried out in triplicate. Error bars indicate standard deviations from the mean value. See also Figure S4 and Figure S5.

Cellular Characterization of Caspase-1 Activity Using Caspase-1 Selective ABP

Having found that CM-269 is highly selective for caspase-1, we investigated in the next step whether the high in vitro selectivity and sensitivity of CM-269 are sufficient for functional cellular studies. Therefore THP-1 and U937 monocytic leukemia cell lines, either nonstimulated or PMA-stimulated, were used as cellular models of monocytes or activated macrophages, respectively (Tsuchiya et al., 1982). Samples were probed for caspase-1 activity, whereas expression and processing of procaspase-1 and its natural substrates proIL-1β and proIL-18 were analyzed by western blotting. With CM-269 caspase-1 activity was detected in both nonstimulated and PMA-stimulated cells with significantly higher activity (2–4-fold, depending on the experiment) detected in PMA-stimulated cells (shown as bars in Figure 4). In contrast, western blot analysis of caspase-1 showed only little differences in its expression and processing upon PMA-treatment (Figure 5A). Processing of proinflammatory proIL-1β by caspase-1 is often used as a read-out of its activity (Scott and Saleh, 2007) as there are no sensitive and selective small-molecule caspase-1 substrates. However, the activity of IL-1β is regulated on many levels, including gene expression (Dinarello, 1997), as various inflammatory stimulators like endotoxins or PMA induce the synthesis of IL-1β (Martinon and Tschopp, 2004). In agreement with the tight regulation of its expression, we were able to detect IL-1β only in PMA-stimulated cells, whereas nonstimulated cells lacked the protein completely (Figure 5B) or its concentration was below detection limit of the western blot. In addition to the proform (p35), lysates of PMA-stimulated cells also contained the processed, mature form (p17) of IL-1β, correlating with the observed caspase-1 activity in PMA-stimulated lysates (bars in Figure 4). Because

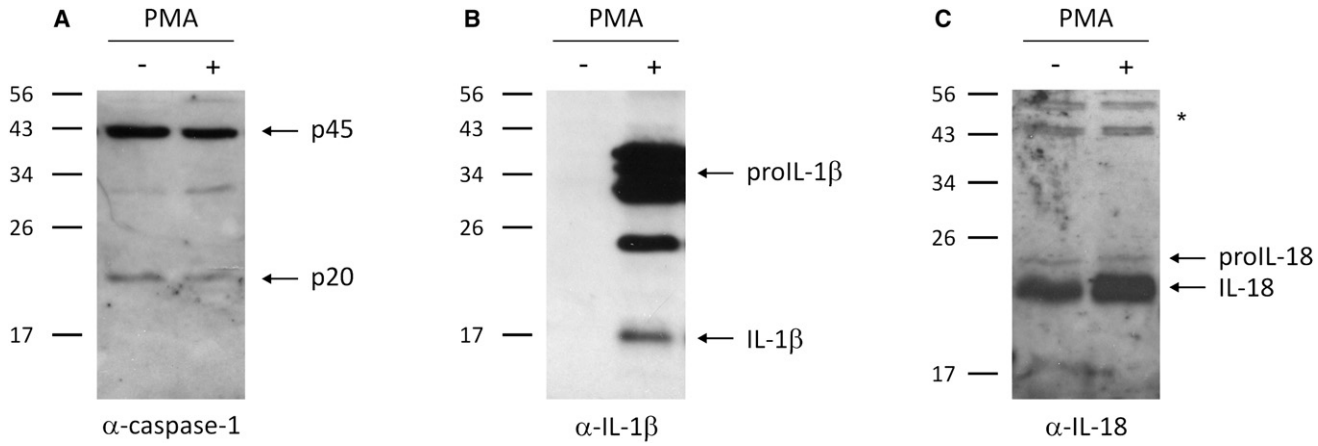

Figure 5. Caspase-1 Processes Its Natural Substrates IL-1β and IL-18

U937 cell lysates of PMA-stimulated and nonstimulated cells were probed with anti-caspase-1 antibody (A; p45, procaspase-1; p20, large subunit of the mature caspase-1), anti-IL-1β antibody (B; p35, proIL-1β; p17, IL-1β) or anti-IL-18 antibody (C; p22, proIL-18; p18, IL-18). Each western blot was carried out at least in triplicate.

nonstimulated cells lacked the IL-1β protein, which precluded us from studying caspase-1 activity indirectly, we probed the lysates for another caspase-1 substrate, IL-18, which is expressed constitutively (Dinarello, 1999). Both nonstimulated and PMA-stimulated U937 cells contained not only the proform (p22) but also the mature form (p18) of IL-18 (Figure 5C), which is in line with the caspase-1 activity toward CM-269 (bars in Figure 4). However, increased levels of the mature form of IL-18 were observed in PMA-stimulated cells (Figure 5C), which may be explained either by the increased expression and/or increased processing due to the higher activity of caspase-1.

Several control experiments were carried out to confirm that the bioluminescent signal stems from active caspase-1. First, the nonspecific cleavage of CM-269 by other caspases or related proteases was tested in PMA-stimulated THP-1 and U937 lysates either by caspase-directed inhibitors or by inhibitors targeting other families of proteases including E-64, a broad-spectrum inhibitor of cysteine cathepsins and calpains, phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, Pepstatin A, targeting aspartic proteases, and ethylenediaminetetraacetic acid (EDTA) as a metalloprotease inhibitor. The two caspase inhibitors included in the panel were the reversible aldehyde Ac-WEHD-CHO, which is known to be very selective for caspase-1 (e.g., ~30,000-fold lower K_i than for caspase-3) and the pan-caspase irreversible inhibitor Z-VAD-fmk, which nonselectively inhibits all the caspases, including caspase-1 (Garcia-Calvo et al., 1998; Rano et al., 1997). Although Z-YVAD-fmk was initially suggested to be a very potent inhibitor of caspase-1, its use was hampered by the finding that it was also efficiently inhibiting cysteine cathepsins, including cathepsin B, in vitro and in intact cells (Rozman-Pungerčar et al., 2003). Therefore, this inhibitor was excluded from our analysis. As shown in Figure 6A, Ac-WEHD-CHO and Z-VAD-fmk were able to efficiently suppress the bioluminescent signal, whereas none of the other inhibitors prevented the hydrolysis of CM-269. Furthermore, similar results were also obtained in nonstimulated U937 lysates (Figure 6B). This suggests that it is caspase-1, and not some of the other proteases, including cysteine cathepsins, calpains, and various metalloproteases, aspartic proteases, and serine proteases, which is responsible for generation of the bioluminescent signal. To completely rule out the influence of proapoptotic caspases, a series of additional experiments were carried out. The fluorogenic substrate Ac-DEVD-AFC, cleaved preferentially by the proapoptotic caspases, was therefore incubated with the lysates of PMA-stimulated and nonstimulated THP-1 and U937 cells (shown as line plot in Figure 4). In contrast to the increased bioluminescent signal of CM-269, no increase in fluorescence could be observed in the lysates of PMA-stimulated cells, suggesting that the more abundant proapoptotic caspases were not activated and therefore did not contribute to the increased signal of CM-269 in this experiment. Finally, the in vivo cleavage of CM-269 by the more abundant proapoptotic caspases was excluded using nonimmune HeLa cells in which apoptosis was induced by ultraviolet (UV) irradiation (Figure 7A). A significant activation of proapoptotic caspases on UV irradiation was observed based on the DEVDase activity (line plot in Figure 7B), which is in agreement with the data from flow cytometry analysis. In contrast, no hydrolysis of CM-269 was observed (bars in Figure 7B), consistent with the high selectivity of the probe for caspase-1.

Having shown that CM-269 enabled monitoring caspase-1 activity in complex proteomic samples, we next wanted to test whether the probe is cell-permeable and therefore amenable for in vivo imaging. PMA-stimulated THP-1 and U937 cells were therefore incubated in the presence of 5 or 10 μM CM-269 for 3 or 5 hr. No bioluminescent signal was observed regardless of the incubation time or the amount of CM-269 added into conditioned media (Figures S6A and S6C). However, when 1 μM (data not shown) or 3 μM CM-269 was subsequently added to cell lysates, a high bioluminescent signal was observed (Figures S6B and S6D), consistent with the probe being non cell-permeable, and ruling out the possibility of caspase-1 inactivation in the samples.

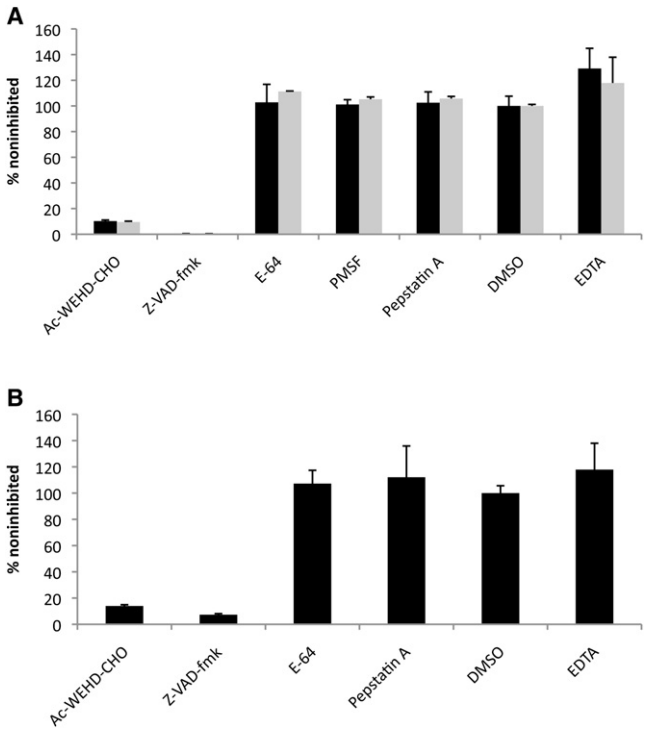

Figure 6. Caspase-1 Inhibitor Ac-WEHD-CHO Suppresses Activity toward CM-269

(A) Cleavage of CM-269 in PMA-stimulated THP-1 (black) and PMA-stimulated U937 (gray) cell lysates was suppressed by the caspase-1 inhibitor Ac-WEHD-CHO (30 μM) and the pan-caspase inhibitor Z-VAD-fmk (30 μM), whereas broad-spectrum inhibitors of cysteine cathepsins (E-64; 30 μM), serine proteases (phenylmethylsulfonyl fluoride [PMSF]; 1 mM), aspartic proteases (Pepstatin A; 1 μM), metalloproteases (ethylenediaminetetraacetic acid [EDTA], 5 mM), or the carrier (dimethyl sulfoxide [DMSO]; 1%) did not inhibit the signal.

(B) Cleavage of CM-269 in nonstimulated U937 cell lysates was suppressed by the caspase-1 inhibitor Ac-WEHD-CHO (30 μM) and the pan-caspase inhibitor Z-VAD-fmk (30 μM), whereas broad-spectrum inhibitors of cysteine cathepsins (E-64; 30 μM), aspartic proteases (Pepstatin A; 1 μM), metalloproteases (EDTA, 5 mM), or the carrier (DMSO, 1%) did not inhibit the signal. Three independent experiments were performed for each panel. Error bars indicate standard deviations from the mean value.

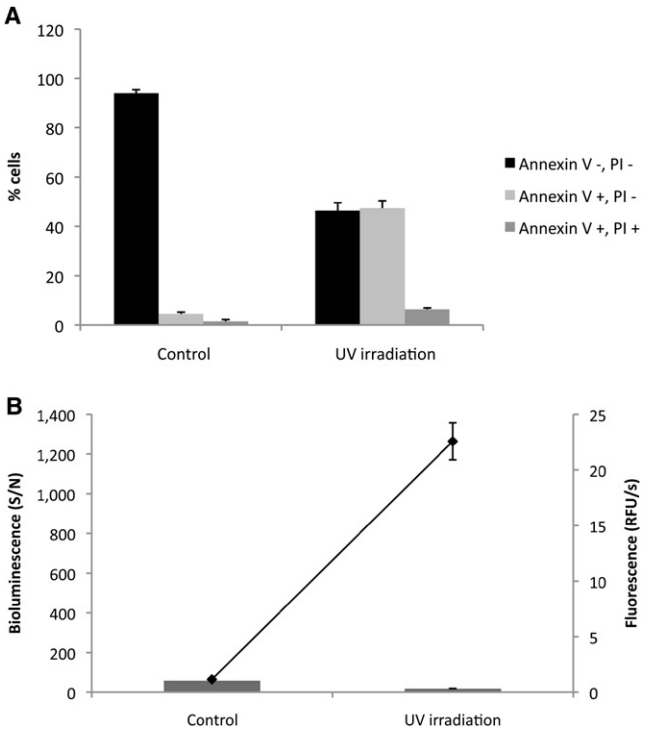

Figure 7. CM-269 Does Not Cross-React with Proapoptotic Caspases in Apoptotic HeLa Cells

(A) Proportion of apoptotic and live HeLa cells irradiated by ultraviolet (UV) light. Annexin V-negative and propidium iodide (PI)-negative cells represent population of live cells. Apoptotic cells are represented as annexin V-positive and PI-negative population of cells. Error bars indicate standard deviations from the mean value.

(B) The bioluminescent signal of CM-269 (3 μM) is shown as bars on the left and the fluorescent signal of Ac-DEVD-AFC (10 μM) as line plot on the right axis. Activity monitoring with Ac-DEVD-AFC showed significant activation of the apoptotic caspases on UV irradiation, whereas CM-269 remained uncleaved. Three independent experiments were carried out. Error bars indicate standard deviations from the mean value.

DISCUSSION

Research in life sciences increasingly requires ABPs that are selective for a particular enzyme and allow monitoring of enzyme activities in a proteomic context. This concept is of particular relevance for protease research, as their specific activation makes direct activity monitoring highly advantageous over detection of enzyme abundance. Until now, small-molecule ABPs for proteases have been designed either as irreversible inhibitors, covalently modifying and inactivating the catalytic site of target enzymes (covalent labeling ABPs) for the subsequent labeling, enrichment and identification of specific enzymes in different physiological samples (Blum et al., 2005, 2007; Evans and Cravatt, 2006), or as peptidic substrates (reviewed in Baruch et al. (2004) and Blum (2008)). Both types of probes exhibit limitations for the monitoring of enzymes in low abundance and with low activity. Covalent labeling ABPs inactivate the enzyme on a single turnover thereby preventing signal amplification. Peptidic substrates offer high cleavage rates, but they frequently suffer from limited selectivity and nonspecific cleavage when applied in proteomic studies. This is particularly relevant for caspases, as cleavage sites of different caspases have largely overlapping motifs, and that is the reason for promiscuous processing of peptidic substrates (McStay et al., 2008). For example, caspase-3, the major executioner caspase, has been shown to cleave the peptidic substrates of other caspases more efficiently than the enzymes to which the substrates were originally designed to (McStay et al., 2008; Pereira and Song, 2008). The highly selective and sensitive monitoring of caspase-1 in complex proteomic samples therefore requires a chemically different type of ABPs, which is (1) not an irreversible inhibitor but a substrate; (2) exhibits exquisite selectivity; and (3) provides high sensitivity in terms of the read-out signal.

In this study, we have applied the *Reverse Design* concept using the scaffold of selective caspase-1 inhibitor Pralnacasan (**1**). Pralnacasan selectively inhibits caspase-1 with an IC_{50} value of 1.3 nM, compared to IC_{50} values of 2.3 μM and 0.12 μM for

caspase-3 and caspase-8, respectively (Linton, 2005). Redesign of the aldehyde warhead of **1** into a caged amino-luciferin-coupled peptide bond provided the bioluminescent CM-269. It has been shown previously that appropriate chemical modification of the 6-amino group of amino-luciferin is an effective way to approach bioluminescent assays for enzymes of interest (Zhou et al., 2008). With regard to kinetic properties, CM-269 was very efficiently cleaved by recombinant caspase-1, with catalytic properties in the range of commercially available, but nonselective fluorogenic substrates (Garcia-Calvo et al., 1999). Moreover, in these in vitro experiments CM-269 exhibited excellent selectivity for caspase-1 with insignificant cleavage by other caspases or by granzyme B. The selectivity of CM-269 was confirmed in complex proteomic samples, because none of the inhibitors targeting serine, aspartic, metallo, or cysteine proteases reduced the CM-269 signal. In our final test of CM-269 selectivity, the probe was added to lysates of apoptotic HeLa cells, which have a high proteolytic potential due to the large amounts of activated proapoptotic caspases, but CM-269 signal did not increase. Collectively, these results demonstrate that CM-269, despite the exchange of the aldehyde warhead with the scissile peptide bond, retained chemically optimized selectivity of the caspase-1 inhibitor Pralnacasan, although the cell permeability was lost. This probe has therefore a significant advantage over the existing fluorescent covalent probes based on the fluoromethylketone warhead (Grabarek et al., 2002), which suffer from the lack of selectivity, and target also other cysteine proteases including the very abundant cysteine cathepsins (Rozman-Pungerčar et al., 2003).

In addition to the excellent selectivity, we have shown that CM-269 exerts high sensitivity. CM-269 was cleaved by recombinant caspase-1 at enzyme concentrations of only about 5-10 pM, which represents a sensitivity increase of about three orders of magnitude compared to Ac-WEHD-AMC, currently the best fluorogenic peptidic substrate for this enzyme (Thornberry et al., 1997). This superior sensitivity proved useful in analyses of complex whole-cell lysates, where activity of caspase-1 could be studied by one-third of the sample required for immunological detection of caspase-1. However, even high amounts (150 μg of total protein) of the same whole-cell lysates were not sufficient to allow monitoring caspase-1 activity with the fluorogenic substrate Ac-WEHD-AMC (data not shown).

In recent years, there has been a major progress toward elucidation of the mechanism of caspase-1 activation in response to specific stimuli, such as various pathogen-associated molecular patterns (lipopolysaccharide, muramyl dipeptide, flagellin from certain Gram-negative bacteria, etc.; reviewed in Franchi et al. (2009)), temperature shift in buffer with a low concentration of potassium (Martinon et al., 2002) or uric acid crystals (Martinon et al., 2006). In these studies, the assembly of large macromolecular complex, termed inflammasome, was identified as a critical step in caspase-1 activation. Generally, the appearance of p20 subunit (large subunit of the mature caspase-1) (Fernandes-Alnemri et al., 2009; Mehta et al., 2001) or N-terminal CARD fragment (Martinon et al., 2002, 2006) has been a read-out for its activation. Although caspase-1 is expressed constitutively and its proform can be readily detected by immunodetection, it is much more difficult to detect its p20 subunit even in stimulated cells due to incomplete processing of the zymogen (Ayala et al., 1994). However, using large amounts of whole-cell lysates we were able to detect the p20 subunit not only in PMA-stimulated but also in nonstimulated cells. This, together with the detectable caspase-1 activity toward CM-269 in nonstimulated cells, raises a question about limited caspase-1 activation in the apparent absence of proinflammatory stimuli. However, in a number of immune cells, the components of inflammasome are expressed constitutively (Fernandes-Alnemri et al., 2009; Martinon et al., 2002, 2006), whereas the minimal composition of the functional inflammasome is still unknown (Faustin et al., 2007). Another argument in favor of active caspase-1 being present in nonstimulated cells is that IL-18, a substrate of caspase-1, is constitutively expressed and processed to the mature p18 form not only in stimulated but also in nonstimulated cells. Upon PMA stimulation, which is taken as a surrogate for the in vivo activation of immune cells, the expression of pro-inflammatory cytokine IL-1β is induced and the expression of IL-18 is upregulated. At the same time, the activity of caspase-1 is increased, which may be explained by the requirement of the cell to process newly expressed cytokines and thereby mount a potent response to a microbial or danger signal.

In conclusion, our results demonstrate that the concept of *Reverse Design* represents an efficient strategy for the design of selective protease ABPs starting from chemically optimized protease inhibitors. Different from the known covalent labeling ABPs, *Reverse Design* ABPs are substrates of the target enzyme, and thus take advantage of signal amplification by the activity of the enzyme of interest. With a sensitive substrate ABP, selective for caspase-1, we have been able to directly monitor and quantify the activity of caspase-1 in complex proteomic samples, such as whole-cell lysates, but not in intact cells. Our results demonstrate that considerable caspase-1 activity is present already in nonstimulated immune cells, which raises questions about activation of caspase-1 in the absence of microbial or danger signals and about potential roles of caspase-1 in physiological processes other than inflammation.

SIGNIFICANCE

Caspase-1 is the major proinflammatory caspase that is critically involved in the activation of proIL-1β and proIL-18, thereby playing a major role in inflammation. Caspase-1, produced as inactive zymogen, gets activated on a multiprotein scaffold, inflammasome, in cells challenged with specific microbial or danger signals. However, complete understanding of caspase-1 activation and its role in inflammation has remained difficult mainly due to the lack of tools to monitor not only its abundance but also its activity. Particularly in complex proteomic samples, selective and sensitive activity-based probes (ABPs) would be advantageous over fluorogenic peptidic substrates and immunodetection.

In the present study, we have applied the concept of *Reverse Design*, where chemically optimized protease inhibitors are turned into selective substrate ABPs (Watzke et al., 2008), to turn the caspase-1 inhibitor Pralnacasan (Siegmund and Zeitz, 2003) into a novel bioluminescent ABP for caspase-1 (CM-269). The probe retained its excellent selectivity and exhibited about three orders of magnitude increased sensitivity compared to the available

fluorogenic peptidic caspase-1 substrates. Moreover, CM-269 offers a significant advantage over the existing fluoromethylketone-based probes that suffer from the lack of selectivity and target also the cathepsins. Its sensitivity allows direct monitoring of caspase-1 activity in complex samples such as whole-cell lysates of monocytes and offers a significant advantage over the traditional, indirect way of monitoring caspase-1 activity based on proteolytic processing of its major substrate, proIL-1β. Using CM-269 we were able to detect caspase-1 activity already in nonstimulated immune cells, raising a question about its physiological role. Therefore, the novel ABP represents a useful and highly sensitive tool to address not only questions related with the role of active caspase-1 in inflammation, but also about the activation of caspase-1 in the absence of microbial or danger signals and about potential roles of caspase-1 in physiological processes other than inflammation.

EXPERIMENTAL PROCEDURES

Synthesis of CM-269

CM-269 was synthesized using a combination of solid-support and solution-phase synthesis as previously described (Watzke et al., 2008). In brief, Fmoc protected 6-amino-D-luciferin **5** (see Supplemental Experimental Procedures, Supplemental Scheme, and Figure S1 for details) was synthesized in solution and coupled via the free carboxy group to the solid support. Likewise the pyridazine building block **8** was prepared as previously published and used after Fmoc-Asp-(Boc)-OH coupling on the solid support. After cleavage from the solid support, the molecule was fully deprotected with 50% TFA/CH_2Cl_2 to yield the final CM-269.

Enzymes

All human caspases were purified according to the published protocols (Garcia-Calvo et al., 1999; Scheer et al., 2006; Stennicke and Salvesen, 1999), which were further optimized. Recombinant human granzyme B was purchased from Alexis Biochemicals (San Diego, CA). Active protein concentrations were determined by active-site titration using the following inhibitors: Ac-YVAD-cmk (Peptanova, Sandhausen, Germany) for caspase-1, -4, and -5, Ac-VDVAD-CHO (Peptanova) for caspase-2, and Ac-DEVD-cmk (R&D Systems, Palo Alto, CA) for caspase-3, -7, and -8.

Coupled Enzymatic Assay for Bioluminescence Measurements

In vitro bioluminescent experiments were carried out in white 96-well COSTAR CellStar plates. The measurements were performed as a coupled enzymatic assay, using QuantiLum recombinant firefly luciferase (Promega, Madison, WI) as reporter enzyme. Bioluminescence was detected with a Tecan Safire2 plate reader in the luminescence mode (λ = 230–850 nm) at 25°C. No signal was observed for uncleaved substrates under standard assay conditions. CM-269, caspases, and luciferase were diluted in 100 mM HEPES buffer, pH 7.4, containing 50 mM $MgSO_4$, 2.5 mM $CaCl_2$, 0.4 mM ATP, 0.6% Prionex, 0.4% Tergitol NP-9, 0.1% silicone antifoam, and 40 mM dithiothreitol (DTT) to the concentrations indicated below. The final dimethyl sulfoxide (DMSO) concentration in the assays did not exceed 1% (v/v).

Kinetic Characterization of CM-269 with Caspase-1

For determination of the kinetic parameters cleavage of the CM-269 by caspase-1 and subsequent oxidation of amino-luciferin by luciferase were carried out consecutively. Caspase-1 (1 nM final concentration) and CM-269 (0.4–25 μM final concentrations) were mixed and incubated.

In two independent experiments an excess of the inhibitor Ac-YVAD-CHO (Bachem, Bubendorf, Switzerland) at a final concentration of 1 mM was added to stop the reaction after 5 min and after 10 min respectively. Concentration of free amino-luciferin produced during the course of caspase-1 cleavage was measured by adding firefly luciferase (1 μg/ml final concentration). The relative light units (RLU) were quantified using an amino-luciferin standard curve (50 nM–1300 nM final concentrations). Kinetic data were fitted by nonlinear least-squares regression analysis according to Equation 1, where υ is the initial velocity, V the limiting rate, K_{ms} the Michaelis constant (considering substrate inhibition), [S] the substrate concentration and K_{is} the substrate inhibition constant:

$$\upsilon = \frac{[S]V_{max}}{\left(K_{ms} + 1 + \frac{[S]}{K_{is}}\right)[S]}. \quad (1)$$

Sensitivity Measurement of CM-269 with Caspase-1

Thirty micromolars CM-269 was mixed with 50 μg/ml (final concentration) firefly luciferase. Reaction was started by adding different concentrations of caspase-1 (5 pM to 10 nM final concentrations). Signal-to-noise (S/N) ratios were calculated according to Equation 2 (Zhang et al., 1999), and the results were plotted on a logarithmic scale to verify the linearity:

$$S/N = \frac{\text{mean signal} - \text{mean background}}{\text{standard deviation background}}. \quad (2)$$

In the fluorescent assay, caspase-1 was diluted in 40 mM PIPES buffer, pH 7.5, containing 100 mM NaCl, 0.1% CHAPS, 10% sucrose, and 10 mM DTT to final concentrations of 0.5–40 nM. The DMSO-dissolved substrate Ac-WEHD-AMC (Peptanova) was added at a concentration of 30 μM, fluorescence measured (λ_{ex} = 360 nm, λ_{em} = 465 nm) and steady-state kinetic data (RFU/s) were taken. The final DMSO concentration in the assay did not exceed 1% (v/v).

Selectivity Measurement of CM-269 with Different Recombinant Human Caspases

Different concentrations of CM-269 (1–130 μM final concentrations) were mixed with recombinant firefly luciferase (10 μg/ml final concentration) and the reaction was started by adding recombinant human caspases and recombinant human granzyme B, all enzymes at 10 nM final concentrations.

The reaction velocity under steady-state condition is given by

$$\upsilon = \frac{V_{max}[S]}{K_m + [S]} = \frac{k_{cat}[E]_t[S]}{K_m + [S]}, \quad (3)$$

for $[S] << K_m$ Equation 3 can be simplified as:

$$\upsilon \approx \frac{k_{cat}}{K_m}[E]_t[S], \quad (4)$$

where k_{cat}/K_m represents the specificity constant.

According to the properties of the bioluminescent measurement, Equation 4 was modified to:

$$S/N \approx \frac{S/N_{max}}{K_m}[E]_t[S], \quad (5)$$

with $(S/N)_{max}/K_m$ becoming the specificity constant. This value can be derived from the slope of the S/N versus $[E]_t[S]$ plot.

PMA-Differentiation of U937 and THP-1 Cells and Western Blot Analysis

U937 and THP-1 cells were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with a 5% CO_2, 95% air atmosphere. Cells were seeded at a density of 0.5 × 10^6 cells/ml in 10-cm culture dishes. Differentiation was induced by 30 ng/ml of phorbol 12-myristate 13-acetate (PMA) over 48 hr. Cells were then grown in the absence of PMA for additional 48 hr. Whole-cell lysates were prepared in 50 mM HEPES buffer, pH 7.5, containing 200 mM NaCl, 10% sucrose, 0.1% CHAPS, 5 mM $MgCl_2$, 0.02% BSA, 0.5% Triton X-100, 1% NP-40, and 100 mM sodium malonate. Equal amounts of whole-cell lysates (75 μg of total cell protein) were applied on a 15% SDS-PAGE, electro-blotted, and probed with anti-caspase-1 antibody (ab17820; Abcam, Cambridge, UK), anti-IL-1β antibody (3ZD; NCI Biological Resources Branch, Bethesda, USA) or anti-IL-18 antibody (ab37640; Abcam). Whole-cell lysates (75 μg of total cell protein) were also measured for activity toward CM-269 (3 μM final concentration) by complementing the buffer with recombinant firefly luciferase (10 μg/ml final concentration) and ATP (0.2 mM final concentration) and toward Ac-DEVD-AFC (Bachem,

10 μM final concentration), to detect the activity of apoptotic caspases. Fluorescence was measured (λ_{ex} = 400 nm, λ_{em} = 505 nm) and steady-state kinetic data (RFU/s) were taken. For bioluminescent measurements, S/N-ratios were calculated as described above.

Inhibition Study of CM-269 in Cell Lysates

PMA-stimulated U937 and THP-1 cell lysates (75 μg of total cell protein) were incubated with 1% DMSO (Sigma), 30 μM inhibitor Z-VAD-fmk (Calbiochem, San Diego, CA), 30 μM Ac-WEHD-CHO (Bachem), 30 μM E-64 (Peptide Institute, Osaka, Japan), 1 mM PMSF (Sigma), 1 μM Pepstatin A (Calbiochem), or 5 mM EDTA (Sigma) for 1 hr at 25°C. The cleavage of CM-269 was determined as described above.

UV-Induced Apoptosis in HeLa cells

HeLa cells were cultured in DMEM with 10% FBS at 37°C in a humidified incubator with a 5% CO_2, 95% air atmosphere. Two days before the experiment, cells were seeded at a density of 5.0 × 10^5 cells/ml into 6-cm culture dishes. UV-irradiation-induced apoptosis was initiated by exposing the cells to UVC light for 45 s. After 18 hr incubation, whole-cell lysates were prepared as described above. Equal amounts of whole-cell lysates (75 μg of total cell protein) were measured for activity toward Ac-DEVD-AFC and CM-269 under the conditions described above.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental Scheme, and six figures, and can be found with this article online at doi:10.1016/j.chembiol.2010.07.011.

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