Studies on the immune system in CLL
Mous, R.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Granzyme B induced apoptosis is enhanced by small molecule inhibitors of XIAP

Rogier Mous¹, Michael Bots³, Margot Jak¹, Mohamed Ahdi¹, René AW van Lier², Eric Eldering², Marinus HJ van Oers¹ and Arnon P Kater¹

Departments of ¹Hematology and ³Experimental Immunology and
³Laboratory of Experimental Oncology and Radiotherapy,
Academic Medical Center, Amsterdam, the Netherlands

Manuscript in preparation
Abstract

Recent studies point out that tumor cells may be protected against cytotoxic T cell attack via increased expression of X-linked inhibitor of apoptosis protein (XIAP). Small molecule inhibitors of XIAP, which have demonstrated their potential as single agents or as enhancers of existing treatment strategies for various types of cancer, may therefore augment immunotherapeutic treatment regimens. To test this hypothesis, we monitored the effect of a sublethal dose of these inhibitors on apoptosis induced by cytotoxic T cells (CTL). First, we observed that XIAP inhibitor 1396-11 enhanced DNA fragmentation induced by recombinant human Granzyme B in Jurkat cells. However, when this inhibitor was used in cytotoxicity and redirected killing assays using P815 or CLL cells as target cells we did not observe synergy between effector cell-induced target cell death and 1396-11. Therefore, our data do not provide a rationale for using small molecule inhibitors of XIAP in CTL-mediated immunotherapeutic approaches.
Introduction

Chronic lymphocytic leukemia (CLL) is a disease that is characterized by a slow accumulation of mature B lymphocytes, which is considered the result of defects in apoptosis. Both changes within death receptor pathways (such as resistance to Fas-mediated apoptosis(23;31)) as well as mitochondrial pathways (predominantly via overexpression of Bcl-2(8;16)) have been implied with decreased sensitivity to apoptosis of CLL cells. Over the past few years, modern drug design has produced agents that specifically target the molecules involved, such as Bcl-2 antisense oligonucleotide oblimersen(30) and various BH-3 mimetics(28;29). These agents all act by activating upstream apoptosis pathways. Nevertheless, actual apoptosis induction can still be prevented downstream by inhibitor of apoptosis proteins (IAPs(21)) that are capable of direct binding to caspase molecules(10). Strikingly, various tumor types have increased expression of IAPs(35) and therefore the threshold for apoptosis induction is raised in these tumor cells. Within the IAP family, especially X-linked inhibitor of apoptosis protein (XIAP) is a crucial player(12) because it directly binds to and thereby prevents activation of caspases-3, -7 and -9(11).

It is therefore no surprise that various therapeutic strategies have been developed for inhibiting the effect of XIAP(13;22;27). Some of these strategies, such as small molecule inhibitors of XIAP(32), might soon be applicable in a clinical setting. These small molecule drugs have been designed to target and inhibit the BIR2 domain of XIAP. Binding of these drugs to XIAP results in the dissociation of caspase-3 from XIAP and as a consequence restoration of caspase-3 activity(36). Importantly, small molecule XIAP inhibitors have already been demonstrated to induce tumor cell death(6;7), to augment the effect of various cytotoxic drugs(32) and also the sensitivity to Fas ligation of tumor cells(18). Interestingly, recent reports also suggest benefit of inhibiting XIAP on CTL mediated cell death(14;17). This finding may have clinical relevance for CLL because CTL-based strategies are the topic of investigation for the development of novel therapies(20) and also the success of allogeneic stem cell transplantation in CLL depends on host-reactive donor-derived CTL(15). Importantly, from the fact that allogeneic stem cell transplantation is also effective in CLL with 17p-(5), it can be deducted that CTL mediated cell death may be at least partially independent of p53. Given that p53 dysfunction is strongly correlated to therapy resistance in CLL(4), these observations warrant the development of active immunotherapeutic strategies against therapy-
resistant CLL. In this perspective, it would be interesting to examine the effect of small molecule XIAP inhibitors on CTL attack.

In the present study, we found that apoptosis of tumor cells induced by recombinant granzyme B (in the presence of recombinant perforin) could be enhanced by pre-incubation with a sub-lethal dose of XIAP inhibitor. We subsequently tested the sensitivity of various tumor cell types to apoptosis-induction by small molecule inhibitors of XIAP and finally tested whether the observed synergy between granzyme B and small molecule inhibitors of XIAP would also result in increased tumor cell death upon CLT attack.

**Methods**

**Cells**

Murine mastocytoma cell line P815, human T cell lymphoma derived J16 cell line (Jurkat) and B cell lymphoma cell line Ramos and Daudi were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM; Gibco) supplemented with 10% v/v Fetal Calf Serum (FCS) and antibiotics. After obtaining informed consent, peripheral blood was drawn from CLL patients. From this material CLL cells were isolated, checked for purity (>90% CD5+/CD19+ cells) via flow cytometry, frozen in 10% DMSO and stored in liquid nitrogen.

**Small molecule inhibitors of XIAP**

Small molecule inhibitors of XIAP were kindly provided by the Torrey Pines Institute for Molecular Studies, San Diego, CA and have been described previously(32). For the experiments, inhibitors 1396-11 (MW 611.8) and 1540-14 (MW 506.4) were used. As a control, the chemically related but inactive compound 1540-20 (MW 515.4) was used.

**In vitro expansion of CMV specific cytotoxic T lymphocytes (CTL)**

PBMC from CMV seropositive CLL patients were used for expansion of CMV-specific CTL as described before(19). Briefly, PBMC (at a final concentration of 5-10x10^6 cells ml^-1) were cultured in IMDM containing 10% v/v Human Pooled Serum (HPS; Cambrex, East Rutherford, NJ), CMVpp65 peptide (1.1 μM) and IL-2 (50 U ml^-1 Biotest Ag, Dreieich, Germany). After one week of culture, cells were re-stimulated every 7 days with CMV peptide loaded irradiated (30 Gy) Epstein Barr virus (EBV) transformed B cell-lines express-
ing either HLA-A2 or HLA-B7 (5x10^4 cells ml^-1) in the presence of IL-2. The percentage of CMV-specific CTL in culture was determined by FACS analysis staining cell culture samples with CD8-PE (Becton Dickinson, San Jose, CA) and APC-conjugated A2 or B7 CMV tetramers (Sanquin, Amsterdam, the Netherlands).

Apoptosis assays
Tumor cell lines or freshly thawed CLL cells were incubated in culture medium with various concentrations of small molecule inhibitors of XIAP. After 6 or 24 hours, the percentage of apoptotic cells was assessed via flow cytometry upon staining with 3,3’-dihexyloxacarbocyanine iodide (DiOC₆(3)) while necrotic cells were detected using propidium iodide (Sigma-Aldrich). All cells that were DiOC₆(3)^bright/propidium iodide^- were considered viable cells, and the percentage of apoptotic cells was calculated as follows: apoptotic cells (%) = 100 – viable cells. In some assays, pan-caspase inhibitor zVAD was added to the tumor cells 30 minutes prior to incubation with XIAP inhibitors. In these assays, besides DiOC₆(3) staining, cleavage of caspase-3 was assessed via intracellular staining (BD Pharmingen, San Jose, CA).

Granzyme B/perforin killing assay
J16 cells were washed 3 times in Hank’s balanced salt solution (HBSS, Invitrogen, Breda, the Netherlands) supplemented with 0.4% bovine serum albumin (BSA) and resuspended in HBSS containing 20mM Hepes buffer (pH 7.2), 2mM CaCl₂ and 0.4% BSA. The cells were then pre-incubated with small molecule inhibitors of XIAP for 30 minutes (final concentration 10μM). Next, sub-lethal doses of recombinant human perforin in combination with different concentrations of recombinant human granzyme B (Sigma, Zwijndrecht, the Netherlands) were added. The cells were then incubated at 37°C for 4 hours. Hereafter, DNA fragmentation was measured by lysing the cells in Nicoletti buffer (0.1% w/v sodium citrate, pH 7.4/0.1% v/v Triton X-100/50μg ml^-1 propidium iodide). Via flow cytometry, DNA content was measured in the nuclei that remained after lysis (sub-G₀/G₁ signal was considered apoptotic).

Redirected killing assay
Freshly isolated PBMC from a healthy donor were stimulated with plate-coated anti-CD3 antibody (16A9T) for 72 hours. These cells were subsequently
used as effector cells in a redirected killing assay. As targets, P815 cells were labeled with CellTrace Far Red DDAO-SE (Molecular Probes, Eugene, OR) to distinguish them from effector cells. The labeled target cells then pre-incubated with small molecule inhibitors of XIAP (final concentration 10μM) for 30 minutes. Next, effector cells were added in a 1:1 ratio and finally, CD3 antibody (1x1) was added in various concentrations. After 4 hours, target cell death was measured in a flow cytometry-based assay by DiOC₆(3) staining. Gating on DDAO-SE positive cells, cell death was measured by determining the percentage of DiOC₆(3)low cells within the target population.

Cytotoxicity assay

CTL activity was measured in a flow cytometry-based assay. As targets, freshly thawed CLL cells were used. CLL cells were first labeled with CellTrace Far Red DDAO-SE. After washing, the labeled CLL cells were incubated at 37°C with CMVpp65 peptide for 1 hour. The labeled target cells were subsequently pre-incubated with small molecule inhibitors of XIAP (final concentration 5μM) for 30 minutes. Autologous CMV-specific effector cells (obtained as described above) were incubated with the labeled target cells at a various effector:target ratios. After 4 hours, target cell death was determined by DiOC₆(3) staining (gating on DDAO-SE positive cells).

ADCC killing assay

J16 cells (stably transfected with PI-9 or mock expression vector)(2) were labeled with DDAO-SE and opsonized with CD45 mAb 15D9 (ascites; dilution 1:300). These cells were then incubated in duplicates in culture medium with effector cells (freshly thawed PBMC from healthy donors) at various effector:target (E:T) ratios for 4 h at 37°C, 5% CO₂. After 4 hours, target cell death was determined by DiOC₆(3) staining (gating on DDAO-SE positive cells). Specific lysis was subsequently calculated as follows: [(% viability of the cells in the control group - % viability of the cells test group)/% viability of the cells in the control group] x 100%.
Results

**XIAP inhibitors synergize with granzyme B induced apoptosis**

It has been described that granzyme B induced apoptosis can be inhibited by overexpression of XIAP(14). To test whether this protective effect can be overcome by treating tumor cells (which frequently overexpress XIAP) with small molecule inhibitors of XIAP, we incubated J16 cells with sub-lethal doses of perforin and various concentrations of granzyme B and evaluated the effect of small molecule inhibitors of XIAP on tumor cell apoptosis (by measuring DNA fragmentation). First, we observed that target cell death induced by granzyme B was dose-dependent (figure 1). However, in the presence of XIAP inhibitor 1396-11, target cell death could be achieved at much lower concentrations of granzyme B compared to conditions in which no XIAP inhibitor or the inactive compound 1540-20 was added. This effect was found to be synergistic, because incubation of target cells with the inhibitors in the absence of granzyme and perforin did not affect cell viability.

![Figure 1. Small molecule inhibitors of XIAP synergize with Granzyme B induced apoptosis.](image)

*Figure 1. Small molecule inhibitors of XIAP synergize with Granzyme B induced apoptosis. J16 cells were treated with sublethal doses of recombinant human perforin (PFN) in combination with various concentrations of recombinant human granzyme B in the presence of XIAP inhibitors. After 4 hours, the cells were lysed and remaining nuclei were stained with propidium iodide to detect fragmented DNA content via flow cytometry (fragmented DNA appears as a sub-G_0/G_1 peak, see methods). The bars represent the mean of three independent experiments, error bars indicate SEM.*
XIAP inhibitors induce apoptosis in various cell types
Small molecule inhibitors of XIAP can induce apoptosis in various tumor cell types(32). Because we wanted to test whether there was a synergistic effect of XIAP inhibitors on cytotoxic T cell (CTL) induced tumor cell death, we assessed the effect of XIAP inhibitors on tumor cell death.

Figure 2. Small molecule inhibitors of XIAP induce apoptosis in tumor cell lines and primary CLL cells. Tumor cell lines and PBMC from a CLL patient were incubated with small molecule inhibitors of XIAP for 24 hours. Subsequently, the percentage of apoptotic cells was assessed via DiOC₆(3)/propidium iodide staining.

A apoptosis induced by XIAP inhibitors in various cell lines.
B the effect of XIAP inhibitors on Ramos cells that either express a dominant negative form of caspase-2 or caspase-9.
C the effect of pre-incubation of Ramos cells with pan-caspase inhibitor zVAD (100μM) on apoptosis induced by XIAP inhibitor 1396-11 (5μM).
D the effect of XIAP inhibitors on PBMC from a CLL patient (95% CD5⁺/CD19⁺ cells).
We observed that both active components 1396-11 and 1540-14 induced tumor cell apoptosis overnight in a dose-dependent fashion in various tumor cell lines (figure 2A), while equivalent concentrations of the inactive compound 1540-20 did not induce tumor cell death. Similar concentrations of the inhibitors did not induce any apoptosis within 4 hours of incubation (data not shown); only a toxic effect was observed at very high concentrations (20μM and higher). Furthermore, the effect of the small molecule inhibitors of XIAP appeared to be independent of caspase-2 or caspase-9 activity, since apoptosis of Ramos cells induced by XIAP inhibitors was not affected by overexpression of dominant negative forms of caspase-2 (DN-2) and caspase-9 (DN-9), respectively (figure 2B). Nevertheless, caspase activity (other than caspase-2 and –9) was found to be essential for XIAP inhibitor induced cell death, because pre-incubation of Ramos cells with pan-caspase inhibitor ZVAD-fmk prevented both mitochondrial depolarization and cleavage of caspase-3 upon incubation with XIAP inhibitors (figure 2C). Finally, primary CLL cells also underwent apoptosis when incubated with XIAP inhibitors (figure 2D).

**XIAP inhibitors do not synergize with CTL mediated cell death**

We tested whether the observed synergy between small molecule inhibitors of XIAP and granzyme B mediated apoptosis might also enhance CTL mediated tumor cell death in an immunotherapy setting. First, we used polyclonally stimulated T cells from a healthy donor as effector cells in a redirected killing assay (see methods). These effector cells were subsequently redirected towards murine mastocytoma P815 cells in the presence of XIAP inhibitors (final concentration 5μM). We observed a small additive effect of the treatment with XIAP inhibitors compared to non-treated tumor cells, but no synergy was observed (figure 3A). Alternatively, we pre-treated CLL cells with XIAP inhibitors (final concentration 5μM), loaded them with CMV immunodominant peptide CMVpp65 and subsequently used these cells as targets for ex vivo expanded autologous CMV specific CTL (see methods). Varying both effector:target ratio and CMVpp65 concentration on the target cells, we did not observe a synergy between CTL mediated tumor cell death and XIAP inhibitors but only a mild additive effect (figure 3B). Since we observed that CLL cells have high expression of PI-9 (data not shown), we tested whether this might be responsible for the absence of synergy between XIAP inhibitors and cytotoxicity. We assessed the effect of PI-9 overexpression on granule-mediated cell death as measured in our assay using PI-9 or mock transduced Jurkat cells.
as targets in an ADCC killing assay. As depicted in figure 3C, overexpression of PI-9 did not impair granule-mediated cell death.

Figure 3. XIAP inhibitors do not enhance CTL-induced target cell death. Cells were pre-incubated for 30 minutes with small molecule inhibitors of XIAP. Subsequently, these cells were used as targets in a CTL killing assay. A The effect of the addition of XIAP inhibitors in a 4 hour redirected killing assay (see methods). The mean of five independent experiments is shown, error bars indicate SEM. B CLL cells (pre-treated with XIAP inhibitors) were loaded with CMVpp65 peptide and subsequently used as targets for in vitro expanded autologous CMV-specific CTL. In this assay, both effector:target ratio and CMVpp65 peptide concentration were varied. C Jurkat cells transfected with a PI-9 or mock expression vector were used as targets in an ADCC killing assay (see methods). The lines represent the average of two duplicate measurements within one experiment; error bars indicate range.

Discussion

In the current study, we tested whether small molecule inhibitors of XIAP enhanced tumor cell death induced by CTL. We observed that, in a sublethal dose, XIAP inhibitor 1396-11 enhanced DNA fragmentation induced by recombinant Granzyme B. However, we only observed a mild additive effect of
XIAP overexpression by tumor cells may be a potent mechanism to prevent caspase activation (35). This is underlined by the results from our study, which demonstrate that treatment with small molecule inhibitors of XIAP 1396-11 or 1540-14 alone induces caspase-dependent cell death. The latter suggests that tumor cells may use overexpression of XIAP as a final threshold to counterbalance the continuous drive to undergo apoptosis (24). Interestingly, the fact that small molecule inhibitors of XIAP have been designed to target the BIR2 domain of XIAP (which is known to selectively bind and block the activity of caspase-3 and –7 (34)) implies that these inhibitors target the intersection of the intrinsic and extrinsic apoptosis route. It is therefore not surprising that these components synergize with various kinds of apoptosis-inducing agents (18;32).

It has been demonstrated that Granzyme B induced target cell death can be antagonized by overexpression of XIAP (14;17). In line with this finding, we here demonstrate that XIAP inhibitor 1396-11 potentiates Granzyme B induced DNA fragmentation in target cells, one of the final steps in apoptosis. Nevertheless, when used in a CTL killing assay, it did not enhance target cell death, which may be explained in several ways. First of all, due to technical limitations we were unable to measure effector caspase activity in the target cells but only assessed target cell death by monitoring the mitochondrial potential loss, a relatively early event in cell death. Alternatively, the synergy between Granzyme B and XIAP inhibitor 1396-11 was established in J16 cells, which express low levels of PI-9, the natural inhibitor of Granzyme B. In contrast, we observed that CLL cells, as well as many other tumor cells (26), express very high levels of PI-9. Therefore, all Granzyme B that enters the CLL cells may be neutralized by PI-9 before exerting its caspase-activating activity. If true, this would also imply that Granzyme B cannot be responsible for CTL induced cell death in CLL. This assumption is supported by our observation that PI-9 overexpressing J16 cells were lysed in a similar fashion as PI-9 low J16 cells (figure 3C). Indeed, there is limited evidence that CTL can induce target cell death independent of Granzyme B in a non-caspase dependent fashion (37). The latter is supported by the observation that target cells treated with pan-caspase inhibitor zVAD-FMK are still effectively lysed by CTL (9;25).

All above-mentioned observations raise the question what mediator(s) secreted by CTL other than Granzyme B might then induce lysis of CLL cells.
Since previous studies from our group demonstrated that CMV specific CTL depend on degranulation to exert their lytic effect(19), perforin seems to be a good candidate. Moreover, perforin is capable of inducing target cell death in the absence of Granzyme A and B(37). Alternatively, other members from the Granzyme family like Granzyme K or H may also fulfill this role(1;3) (although the latter is mainly found in NK cells(33)). Importantly, all above-mentioned molecules exert their cytolytic function in a caspase-independent fashion, so that overexpression of IAPs will not protect tumor cells from the lytic activity of these molecules.

In summary, our study demonstrates that small molecule inhibitors of XIAP enhance Granzyme B induced apoptosis but paradoxically do not induce increased target cell death when used in in vitro killing assays. It therefore raises the question whether Granzyme B can be held responsible for killing of CLL cells (which in general have high expression of PI-9) by CMV specific CTL. Nevertheless, our study also confirms the strong cytotoxic potential of these CTL as mediators of active immunotherapy for CLL.
Chapter 6

Reference List


XIAP inhibitors and cytotoxicity


Chapter 6


