Leukemia Research 38 (2014) 383–392

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Ritonavir, nelfinavir, saquinavir and lopinavir induce proteotoxic stress in acute myeloid leukemia cells and sensitize them for proteasome inhibitor treatment at low micromolar drug concentrations

Marianne Kraus[a], Hendrik Müller-Ide[b], Thomas Rückrich[b], Jürgen Bader[a], Herman Overkleeft[c], Christoph Driessen[a,b,*]

[a] Department of Oncology and Hematology, Cantonal Hospital, St. Gallen, Switzerland
[b] Department of Medicine II, University of Tübingen, Germany
[c] Leiden Institute of Chemistry, University of Leiden, The Netherlands

ARTICLE INFO

Article history:
Received 27 February 2013
Received in revised form
25 November 2013
Accepted 14 December 2013
Available online 25 December 2013

Keywords:
Unfolded protein response
Acute myeloid leukemia
Proteasome inhibitions
HIV protease inhibitor

ABSTRACT

Background: Protein metabolism is an innovative potential therapeutic target for AML. Proteotoxic stress (PS) sensitizes malignant cells for proteasome inhibitor treatment. Some HIV protease inhibitors (HIV-PI) induce PS and may therefore be combined with proteasome inhibitors to achieve PS-targeted therapy of AML.
Methods: We investigated the effects of all nine approved HIV-PI alone and in combination with proteasome inhibitors on AML cell lines and primary cells in vitro.
Results: Ritonavir induced cytotoxicity and PS at clinically achievable concentrations, and induced synergistic PS-triggered apoptosis with bortezomib. Saquinavir, nelfinavir and lopinavir were likewise cytotoxic against primary AML cells, triggered PS-induced apoptosis, inhibited AKT-phosphorylation and showed synergistic cytotoxicity with bortezomib and carfilzomib at low micromolar concentrations. Exclusively nelfinavir inhibited intracellular proteasome activity, including the β2 proteasome activity that is not targeted by bortezomib/carfilzomib.
Conclusions: Of the nine currently approved HIV-PI, ritonavir, saquinavir, nelfinavir and lopinavir can sensitize AML primary cells for proteasome inhibitor treatment at low micromolar concentrations and may therefore be tested clinically toward a proteotoxic stress targeted therapy of AML.

1. Introduction

The prognosis of aged patients (>60 y) with AML is still dismal. Although a proportion of reasonably fit patients with AML can be cured with allo PBSCT at least until the age of 70, the median survival of elderly patients treated with intensive approaches is approximately eight months. Thus far, the most promising approach for the majority of AML patients >60 years that cannot undergo intensive treatment approaches are low-intensity cytarabine, 5-azacytidine, and decitabine. The latter results in >20% complete responses, however, median survival is still only 7.7 months [1]. Thus, traditional chemotherapy approaches that induce genotoxic stress by targeting cell cycling and the DNA replication machinery are often ineffective in this patient population. The elimination of malignant cells via induction of proteotoxic stress (PS) is emerging as an innovative alternative strategy for AML [2,3]. PS occurs when misfolded or dysfunctional protein accumulates in the endoplasmatic reticulum [4]. PS is counteracted by the unfolded protein response (UPR) via three independent signaling pathways, PERK, ATF6, and IRE1. The UPR is a complex response that decreases the rate of protein biosynthesis, while increasing the capacity for protein folding and destruction via upregulation of chaperones (BiP or other heat shock proteins (HSPs), the protein disulfate isomerase PDI) as well as the proteasome [5]. Excess activation of the UPR results in UPR-induced apoptosis [4]. Due to their genetic instability, rapid growth and critical metabolic substrate supply, malignant cells are more susceptible to killing through the manipulation of protein homeostasis, resulting in cancer preferential vulnerability [6,7]. The biology of AML cells is characterized by the accumulation of somatic mutations in highly proliferative hematopoietic progenitors, suggesting a state of proteotoxic stress in AML cells.

* Corresponding author at: Onkologie/Hämatologie, Kantonsspital, St. Gallen, Rorschacherstrasse 95, CH-9007 St. Gallen, Switzerland. Tel.: +41 71 494 1162.
E-mail address: christoph.driessen@kssg.ch (C. Driessen).

0145-2126/$ – see front matter © 2014 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.leukres.2013.12.017

Key effectors of the UPR are commonly activated in AML, and UPR activation may be directly involved in the suppression of myeloid differentiation in AML patients [8,9]. The combination of proteasome inhibition (increasing the *afterload* of the ER) with direct UPR induction (increasing the *preload* of the ER) in malignant cells significantly increases UPR activation and leads to subsequent UPR-induced apoptosis, in contrast to either strategy alone, and is referred to as "proteotoxic stress targeted therapy" (PSTT) [6,7,10].

HIV protease inhibitors (HIV-PI; ritonavir, lopinavir, saquinavir, nelfinavir, darunavir, atazanavir, amprenavir, indinavir, tipranavir) are orally available drugs approved for HIV treatment. Besides inhibiting their viral target, the HIV protease, the first generation HIV-PI ritonavir, nelfinavir, indinavir and saquinavir induce ER stress in eukaryotic cells and have shown activity in various preclinical models of hematologic malignancies, including acute leukemia, in vitro and in vivo [11–16], so that they are currently being repositioned as potential antineoplastic drugs [17,18]. The mechanism of ER stress induction in malignant cells by HIV-PI is still controversial and may involve direct proteasome inhibition [16,19], as well as disruption of the UPR pathway, either via direct inhibition of the S2P protease that regulates proteolytic activation of ATF6 [20,21], or via interfering with the function of heat shock proteins, namely HSP90 [22].

Although proteasome inhibition selectively eliminates leukemic stem cells in preclinical models in vivo [23], the proteasome inhibitor bortezomib has so far not shown clinical activity against AML. Ritonavir and nelfinavir sensitize sarcoma, NSCLC and multiple myeloma for proteasome inhibitor treatment in vitro and in vivo [24]. It is unknown, whether HIV-PI can likewise be used to sensitize AML cells for proteasome inhibitor treatment. Moreover, the first generation HIV-PI have been replaced by second generation drugs (lopinavir, atazanavir, amprenavir, atazanavir, darunavir) with improved tolerability and pharmacokinetic properties. To allow the selection of the most appropriate HIV-PI for clinical testing in combination with proteasome inhibitors as part of a PSTT-directed AML treatment, and to estimate HIV-PI serum concentrations presumably required for antileukemic activity of such a combination, we here compare the potential antileukemic and proteasome inhibitor-sensitizing effects of all approved HIV PI in vitro.

2. Methods and materials

2.1. Cells and inhibitors

Human monocytic leukemia cell lines THP-1, HL-60 and U937 were obtained from ATCC. Amprenavir, atazanavir, darunavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir and tipranavir were provided by the NIH AIDS Reagent Program. Cell culture and treatment was performed as in [25,26]. 5×10^6 cells were used per sample and inhibitors were added simultaneously at the start of the experiment for its entire duration. Bortezomib, carfilzomib and the vinylsulfone-type proteasome inhibitor NLVS [27,28] were synthesized at the Leiden Institute of Chemistry.

2.2. MTT assay, western blot, antibodies

The CellTiter 96® (Promega) was used to determine cytotoxicity [26] for cell lines. 5×10^4 cells were seeded per 96well and incubated for 48 h after addition of the respective inhibitors. Results represent mean values from quadruplicate wells in one of at least three independent experiments. Cell viability in primary cells (5×10^3/well) was measured with CellTiterGlo® assay (Promega) in an otherwise identical experimental setup. SDS-PAGE and western blot was performed as described with the same set of antibodies as in [26].

2.3. Determination of proteasome activity by active-site labeling

The proteasome-specific affinity probe Bodipy TMR-Ahx_3L_3VS (MV-151) was synthesized and used as described [29,26]. Proteasome subunit-specific fluorescence signals (separately for β2/2i and β1/1i/5/5i) were quantified using Bio 1D software (Vilber Lourmat).

2.4. Colony formation assay

5×10^4 cells/ml were seeded per 24well (ultra low attachment surface (Corning)) in MethoCult H4034 Optimum medium (Stemcell Technologies) according to the manufacturer's suggestion, in the presence/absence of the respective inhibitors, and cultivated for 2 weeks, followed by counting of colonies.

2.5. Patient samples

Patient samples were obtained after approval by the independent ethics review board and written informed consent, in accordance with ICH-GCP and local regulations. Leukemia cells were retrieved from peripheral blood of untreated patients and enriched by Ficoll density centrifugation to a purity of more than 80%, where necessary. 5×10^6 cells were seeded per sample, as described [25], and inhibitors were added simultaneously at the start of the experiment for its entire duration. Monocytes were enriched from PBMC to >80% purity using a percoll gradient [30].

2.6. Statistical analysis

Unless stated otherwise, one representative experiment out of at least 3 independent experiments is shown; for MTT assays mean values from quadruplicate samples are represented. Synergism between bortezomib and the different HIV-PI was calculated using Combination Index described in [31]. A combination Index (CI) <1 indicates synergism, >1 indicates antagonism. Normalized isobolograms were produced by plotting the bortezomib ratio (monotherapy dose vs. dose needed in combination to reach the same effect) on the *x*-axis versus the HIV-PI ratio on the *y*-axis. The statistical significance for different IC_{50} values was calculated using Student's *t*-test.

3. Results

3.1. Cytotoxic effect of alternative HIV-PI on AML cells

Incubation of AML cell lines (THP-1 and HL-60) or primary AML cell samples with each of the currently approved HIV-PI resulted in a dose-dependent cytotoxic effect of lopinavir, nelfinavir, ritonavir, or saquinavir (LNRS-HIV-PI), in contrast to amprenavir, atazanavir, darunavir, indinavir and tipranavir (Fig. 1A). The IC_{50} values against primary AML cells were similar for each of the LNRS-HIV-PI, and in the 20 μM range, in contrast to the remaining HIV-PI. Interestingly, also bortezomib-resistant HL-60 cells (HL-60a cells, adapted to survive in the presence of bortezomib 80 nM [32]) showed a pattern of sensitivity and dose response against HIV PI-induced cytotoxicity that was comparable to its non-adapted parental cell line (HL-60wt), suggesting that cytotoxicity of HIV-PI is not mainly induced by proteasome inhibition.

We further tested the cytotoxic effect of ritonavir, the lead HIV-PI drug, on primary patient cell samples (AML and ALL), in comparison to AML cell lines (HL-60, THP–1, U937) and primary cells from healthy donors (PBMC and monocytes from three