Leukemia Research

ELSEVIER

Research
CLINICAL AND LABORATORY STUDIES

The NO-modified HIV protease inhibitor as a valuable drug for hematological malignancies: Role of p70S6K

CrossMark

Danijela Maksimovic-Ivanic [a], Marija Mojic [a], Mirna Bulatovic [a], Milica Radojkovic [b], Milos Kuzmanovic [c], Slobodan Ristic [b], Stanislava Stosic-Grujicic [a], Djordje Miljkovic [a], Eugenio Cavalli [d], Massimo Libra [d], Paolo Fagone [d], James McCubrey [e], Ferdinando Nicoletti [d,*], Sanja Mijatovic [a]

[a] Department of Immunology, Institute for Biological Research "Sinisa Stankovic", Belgrade University, Belgrade, Serbia
[b] Clinical Center "Dr Dragisa Misovic", Medical Faculty, University of Belgrade, Belgrade, Serbia
[c] Institute for Health Care of Mother and Child of Serbia "Dr Vukan Cupic", Medical Faculty, University of Belgrade, Belgrade, Serbia
[d] Department of Biomedical Sciences and Biotechnology, University of Catania, Catania, Italy
[e] Department of Microbiology and Immunology, Brody School of Medicine at East Carolina University, Greenville, NC 27858, USA

ARTICLE INFO

ABSTRACT

Covalent attachment of NO to the first approved HIV protease inhibitor Saquinavir (Saq-NO) expands the therapeutic potential of the original drug. Apart from retained antiviral activity, the modified drug exerts strong antitumor effects and lower toxicity. In the present study, we have evaluated the sensitivity of different hematological malignancies to Saq-NO. Saq-NO efficiently diminished the viability of Jurkat, Raji, HL-60 and K562 cells. While Jurkat and Raji cells (established from pediatric patients) displayed abrogated proliferative potential, HL-60 and K652 cells (originated from adults) exposed to Saq-NO treatment underwent caspase dependent apoptosis. In addition, similar sensitivity to Saq-NO was observed in mononuclear blood cells obtained from pediatric patients with acute lymphoblastic leukemia (ALL) and adult patients with acute myeloid leukemia (AML). Western blot analysis indicated p70S6 kinase as a possible intracellular target of Saq-NO action. Moreover, the addition of a NO moiety to Lopinavir resulted in improved antitumor potential as compared to the parental compound, suggesting that NO-derived HIV protease inhibitors are a potential new source of anticancer drugs with unique mode of action.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Over the last few decades many discoveries have been achieved in the field of cancer therapy, and in particular in the area of small molecule inhibitors of oncogenic cell signaling pathways [1]. Unfortunately, therapy failure due to relapsed or primary resistant disease is still frequent and is associated to severe toxicity and side effects [2]. Thus, further studies are warranted to identify novel anticancer compounds with similar mode of action but with lower toxicity. Drugs used for the treatment of viral infection such as HIV protease inhibitors (HPIs) may represent a promising therapeutic option in the cancer setting [3]. Their anticancer properties were incidentally noticed when it was observed that patients exposed to

HPIs, exhibited significantly lower rate of HIV associated cancers [3]. Independently of HIV protease inhibition, these drugs reduce angiogenesis, cell invasion and viability of malignant cell through induction of apoptosis/autophagy, accompanied with Akt inhibition [4–11]. Beside their effectiveness on solid cancers, HPIs were found efficient in the treatment of blood cancers [12–14]. Ritonavir induced apoptosis and inhibited NF-kB activity in adult T cell leukemia. Ritonavir, Saquinavir (Saq) and Nelfinavir through their inactivation of STAT3 and ERK1/2 led to growth arrest and apoptosis of human multiple myeloma cells [15]. Nelfinavir was also found to induce mitochondria independent apoptosis of leukemia cell lines and impaired proteasome activity and proliferation of multiple myeloma cells in vitro and in vivo [16]. Importantly, Saq displayed antiproliferative activity even against imatinib-resistant chronic myelogenous leukemia cell lines [6]. Beside direct effects, HPIs are able to potentiate the effect of 1,25-dihydroxyvitamin D3 or ATR on myelioid leukemia cells [14,17]. Recently, Kraus et al. [12] demonstrated that Nelfinavir augments proteasome inhibition by

Bortezomib and overcomes Bortezomib and Carfilzomib resistance [12]. Limitations of the usage of HPIs are toxicity and development of resistance. Different chemical manipulation like packaging in nanostructured lipid carriers [18] has been examined to reduce toxicity. We focused our attention on a derivative of Saq obtained by the linkage of the NO moiety as NO-hybridization has found useful to increase the anticancer potential in the case of nonsteroidal anti-inflammatory drugs [19]. Saquinavir-NO (Saq-NO) was found to be significantly superior than Saq in diminishing the viability of a variety of adherent tumor cell lines in vitro and abrogating the growth of melanoma, prostate and colon cancer in syngeneic and xenograft models in vivo [20–25]. Although in p53 deficient and iNOS positive cell lines, Saq-NO induced apoptotic cell death, its antitumor action was mostly executed through blockade of cellular proliferation of cancer cells and reversion to normal phenotype through process of differentiation or transdifferentiation [20–22]. Additional benefit of this drug is its potential to sensitize tumor cells to chemotherapeutics and antitumor immune responses [22,25–27]. We also demonstrated that the mechanism of action of Saq-NO is partly based on the property to inhibit the activation of p70S6K protein that is important for many cellular processes, transcription, translation, protein and lipid synthesis, cell growth and metabolism [25].

In this paper, we have studied the effects of Saq-NO on different blood cancers. We found that Saq-NO efficiently suppressed the expansion of lymphoma and leukemia cell lines in vitro. Moreover, Saq-NO decreased the viability of malignant cells isolated from patients with acute lymphoblast leukemia and acute myeloid leukemia. To the best of our knowledge, this is the first evidence of efficacy of modified HIV protease inhibitors against blood cancers.

2. Materials and methods

2.1. Reagents

Fetal calf serum (FCS), RPMI-1640, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), and propidium iodide (PI) were attained from Sigma (St. Louis, MO). Annexin V-FITC (AnnV) was from Biotium (Hayward, CA). Acridine orange (AO) was from Labo-Moderna (Paris, France). The Jurkat, Raji, HL-60 and K562 cell lines were purchased from ATCC. Cells were routinely maintained in HEPES-buffered RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 0.01% sodium pyruvate, and antibiotics (culture medium) at 37 °C in a humidified atmosphere with 5% CO_2. Cells were seeded at 1×10^4/well in 96-well plates for viability determination and 2.5×10^5/well in 6-well plate for flow cytometry.

2.2. Isolation of peripheral blood mononuclear cells (PBMC)

Heparinized blood was obtained from ALL and AML patients before chemotherapy treatment and healthy volunteers, centrifuged at $115 \times g$ 10 min and plasma were layered on 4 ml lymphocyte separation medium. Samples were centrifuged for 20 min at $800 \times g$ without brake and the interface layer ring was collected carefully. Cell suspension was washed twice in PBS 10 min at $700 \times g$. Finally, cells were resuspended in RPMI-1640-10% FCS. Cells were counted in Turk solution while trypan blue staining was used for viability judgment. The study was approved by the Ethics Review Board of Clinical Center "Dr Dragisa Misovic" (01-10000/6) and Institute for Health Care of Mother and Child of Serbia "Dr Vukan Cupic" (970/3). Cell samples from humans were obtained after written informed consent.

2.3. Acidic phosphatase assay

Cells (1×10^5 cells/well) in 100 μl of culture medium were cultivated in the presence of a wide range of concentrations of tested drugs for 48 h. At the end of treatment, the cells were incubated in the presence of the substrate for acidic phosphatase (1.1 mg/ml p-nitro-phenyl-phosphate, 0.4% Triton-X, 0.3% Na-acetate) for 2 h at 37 °C. The reaction was terminated by the addition of 50 μl 1.3 M NaOH. The absorbance of developed dye was read at 405 nm by using an automatic microplate reader (LKB) [28].

2.4. Cell cycle analysis

The cells (2.5×10^5/well) were exposed to the IC_{50} doses of each NO-modified drugs and equal dose of parental drug for 48 h, then fixed in 70% ethanol at 4 °C over night. After washing in PBS, the cells were incubated with PI (20 μg/ml) and RNase (0.1 mg/ml) for 30 min at 37 °C in the dark [20]. Red fluorescence was analyzed with

FACS Calibur flow cytometer (BD, Heidelberg, Germany). The distribution of cells in different cell cycle phases was determined with Cell Quest Pro software (BD).

2.5. Annexin V-FITC/PI and apostat staining

The cells (2.5×10^5/well) were treated with IC_{50} dose of each compound or equal dose of the parental drug for 48 h, then stained with AnnV-FITC/PI (Biotium, Hayward, CA) or apostat (R&D Systems, Minneapolis, MN USA) following the manufacturer's instructions. Cells were analyzed with FACS Calibur flow cytometer (BD, Heidelberg, Germany) using Cell Quest Pro software (BD).

2.6. CFSE staining

The cells were stained with 1 μM of carboxyfluoresceinsuccinimidyl ester (CFSE) for 10 min at 37 °C, then washed and treated with IC_{50} dose of the tested compounds for 96 h. At the end of cultivation, the cells were washed and analyzed with FACS Calibur flow cytometer [20].

2.7. Western blot analysis

The cells were treated with an IC_{50} dose of Saq-NO and equal dose of Saq for 2, 6, 12 and 18 h and lysed in protein lysis buffer containing 62.5 mM Tris–HCl (pH 6.8 at 25 °C), 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% (w/v) bromophenol blue. The samples were then electrophoretically separated on 12% SDS-polyacrylamide gel. PageRuler prestained ladder (Thermo scientific, USA) was used as protein molecular weight marker. Electrotransfer to polyvinylidene difluoride membranes at 5 mA/cm² was done using a semidry blotting system (Fastblot B43; Bio-Rad, Goettingen, Germany). The membranes were blocked with 5% (w/v) BSA in PBS with 0.1% Tween 20, and then blots were incubated with specific antibodies to p-S6 (Ser240/244), S6, p-p70S6K (Thr389), p-p70S6K (Thr421/Ser424), p70S6K and tubulin (Cell Signaling Technology, Danvers, MA), followed by incubation with secondary antibody (ECL donkey anti-rabbit horseradish peroxidase-linked; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Bands were visualized using a chemiluminescence detection system (ECL; GE Healthcare).

2.8. Bioinformatical analysis of primary sequence homology

Amino acid sequences of desired proteins were obtained from NCBI PubMed database. Homology region in protein primary sequence was analyzed using BLASTP 2.2.28 program [29,30].

2.9. Statistical analysis

The results of cellular viability are presented as means ± SD from three independent experiments. The significance of the differences between various treatments was assessed by analysis of variance (ANOVA), followed by a Student–Newman–Keuls test. Two-tailed matched pair Student's *t* test was performed to compare effects of Saq and Saq-NO on primary cancer cell lines, Mann–Whitney test was used to compare the effect of Saq and Saq-NO on pediatric vs adults patients. A *p* value less than 0.05 was considered significant.

3. Results

3.1. Saq-NO decreased the viability of blood cancer cell lines

In order to test the efficacy of Saq-NO in blood cancers, four different human cell lines were chosen: T lymphoblastic leukemia – Jurkat, Burkitt's lymphoma – Raji, erythroleukemia – K562 and promyelocytic leukemia HL-60. The cells were exposed to a range of concentrations of Saq and Saq-NO and after 48 h their viability was measured by acidic phosphatase test. Both drugs exerted marked antitumoral effects in all the cell lines tested (Fig. 1). Saq-NO showed significant lower IC_{50} values than Saq with the lowest IC_{50} obtained on HL-60 cells. Sensitivity of blood cancer cell lines to Saq-NO were even higher than previously identified in adherent tumor cell lines [20,21,23,25]. Interestingly, for Jurkat, Raji and K562 cells, higher concentrations of Saq resulted more efficient than similar concentration of Saq-NO, while the opposite situation is observed at lower concentrations. Indeed, in contrast to the results observed with HL60 cells, a plateau effect was observed upon the treatment of Jurkat, Raji and K562 cells with Saq-NO, indicating that further dose enhancement of Saq-NO was not in correlation with the decrease of cell viability.
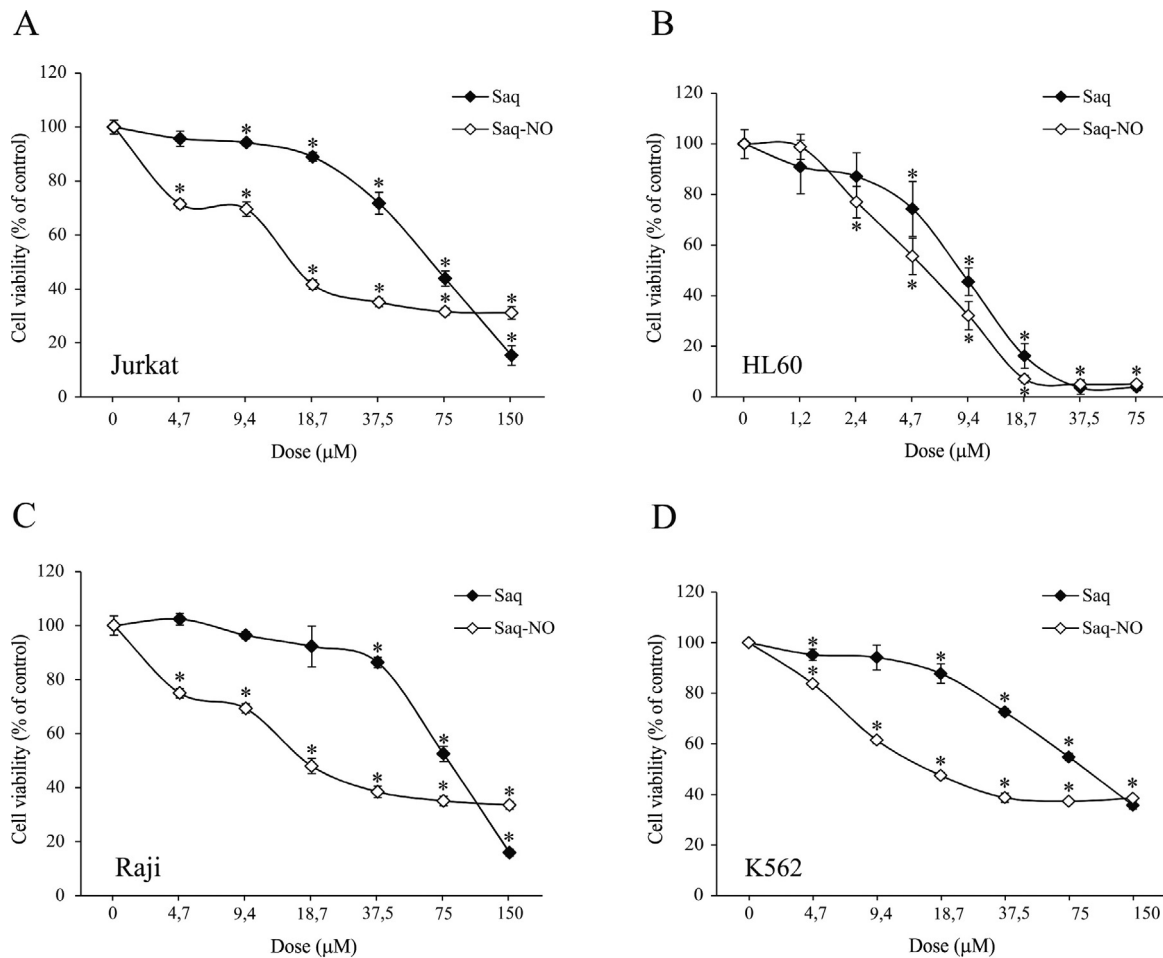
Fig. 1. Saq-NO diminished the viability of blood cancer cells. Cells were exposed to various concentrations of tested drugs and after 48 h, cell viability was determined by acidic phosphatase assay. The data are presented as mean ± SD from three independent experiments. *$p < 0.05$, refers to controls.

3.2. The modality of action of Saq-NO is defined by cell specificity

Our previous data demonstrated that Saq-NO primarily displayed cytostatic potential against tumor cells. To evaluate the mechanism responsible for the decreased viability, cell cycle analysis was performed after 48 h of treatment with equimolar concentrations of Saq-NO and of parental drug. As seen in Fig. 2A, Saq-NO treatment was associated with an increased percentage of hypodiploid subpopulation in HL-60 and K562 cells as compared to Saq. Only insignificant percentage of apoptotic cells were observed in Jurkat and Raji cells upon treatment with both Saq-NO and Saq. Since the lower number of viable cells upon the treatment might be a consequence of suppressed proliferation, these cells were stained with CFSE and analyzed after 96 h of incubation with Saq or Saq-NO (Fig. 2B). While both control and Saq-treated cultures almost completely divided, less than 20% of cells retained their proliferative capacity after exposure to Saq-NO. A similar effect was seen for K562 and HL-60 cells where upon treatment with Saq-NO only 6.8% and 4.6% of cells, respectively, proliferated (as compared to the 80% and 95% of divided control cells, respectively). Annexin/PI staining demonstrated that Saq-NO treatment of HL-60 and K562 cells resulted in augmented percentage of both early and late apoptotic cells confirming that the drug triggered the apoptotic process in these cells (Fig. 2C). Accordingly apostat staining detected caspase activation in both HL-60 (mean fluorescence 31.8 vs. 16.3 as compared to control cells) and K562 cells (mean fluorescence 39.5 vs. 22.2 as compared to control cells). On the other hand, treatment of Jurkat and Raji cells with Saq-NO led to insignificant apoptosis

(4.8% of early and 1.4% of late apoptotic vs. 3.7% and 1.0% in control Jurkat cells; 5.3% of early and 1.9% of late apoptotic vs. 2.5% and 0.5% in control Raji cells). Taken together, these data support the concept that cell specificity defines the mode of the drug action.

3.3. Saq-NO decreased the viability of mononuclear blood cells isolated from the patients with ALL and AML

The capacity of Saq-NO to reduce the viability of malignant cells obtained from untreated pediatric patients with T lymphoblastic leukemia (T-ALL) and untreated adult patients with acute myeloid leukemia (AML) were determined. The cells were isolated from peripheral blood and were examined in the same experimental conditions as previously described. IC_{50} values for Saq-NO and Saq in children (Table 1) and adult (Table 2) cell cultures were higher than those observed in corresponding cell lines, but were lower than the IC_{50} values obtained for the blood of healthy volunteers ($53.15 \pm 14.26\,\mu M$, $n = 4$). Importantly, Saq-NO displayed higher activity against patients' blood cells in comparison to the original drug ($p = 0.02$ for adults and $p = 0.05$ for pediatric patients). Interestingly, Saq-NO was equally effective on the cells obtained from patients sensitive and resistant to steroid therapy, which is an indicator of bad prognosis (Table 1). Higher efficacy of Saq-NO against adults (average $IC_{50} = 17.6 + 12.6$) in comparison to pediatric (average $IC50 = 33.2 + 19.7$) patients ($p = 0.06$) is probably due to its potential to abolish the malignant cells resistance to apoptosis which is one of the hallmarks of adult acute myeloid leukemia [31] (Table 2).
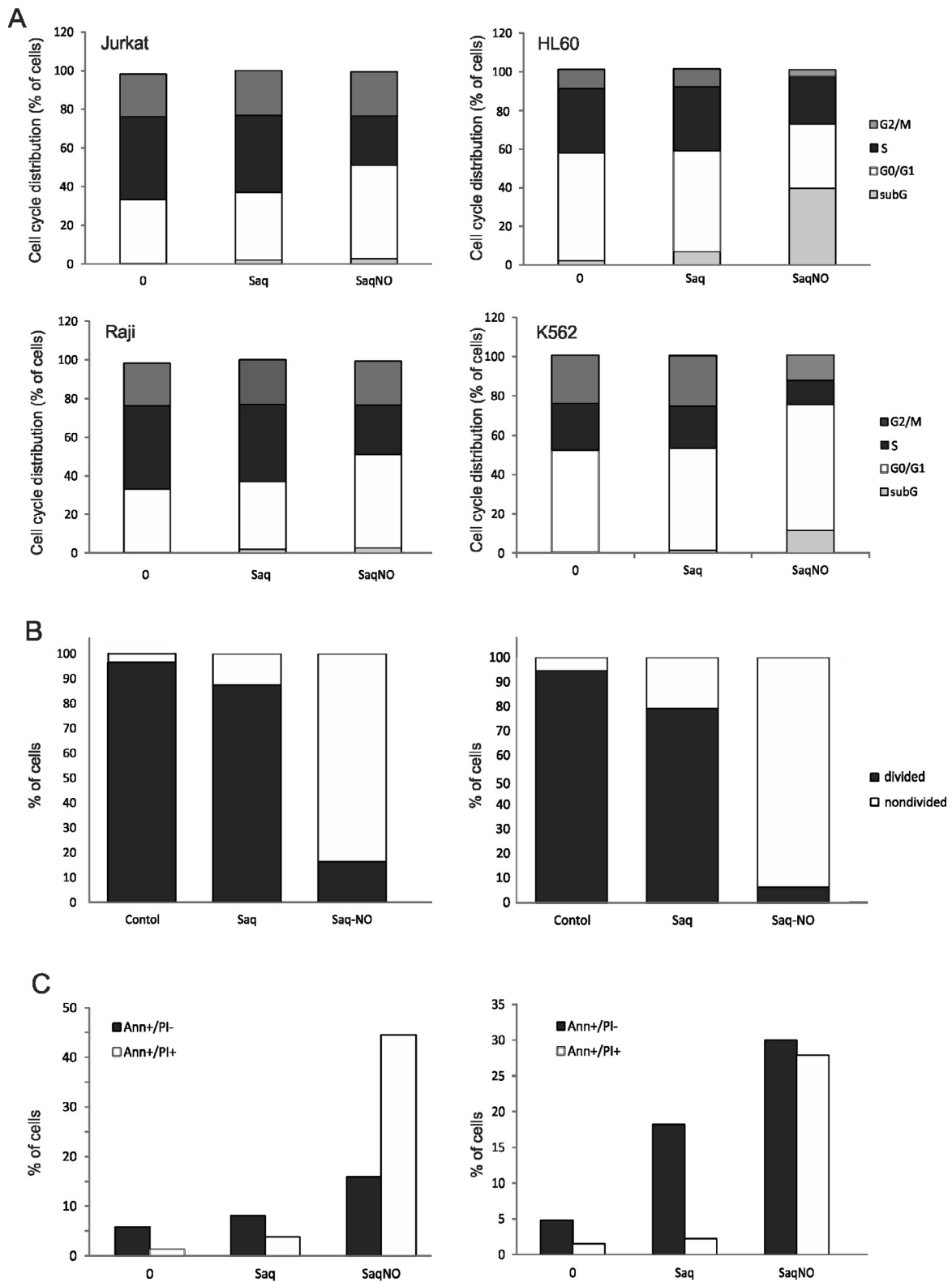
Fig. 2. Effects of Saq-NO in blood cancer cell lines. Cells were treated to IC$_{50}$ dose of Saq-NO and equal dose of Saq and after 48 h cell cycle analysis was performed (A), alternatively CFSE stained Jurkat (left) and Raji (right) cells were analyzed after 96 h of treatment (B). HL-60 (left) and K562 (right) cells were stained by Ann/PI upon 48 h of treatment (C).

3.4. Saq-NO permanently inhibited p70S6 kinase expression

Saq is designed as a peptidomimetic drug created to block the proteolytic activity of the HIV protease and prevent formation of

infective viral particles [3]. Subsequently, it was discovered that Saq and Saq-NO affected mammalian cell physiology, indicating their ability to functionally modulate different intracellular molecules. To predict possible interactions between Saq-NO and key

Table 1
Characteristics of ALL patients and effects of Saq and Saq-NO on isolated PBMC.

Sex/age	Disease	Risk	WBC × 10^9/l	Blasts (%)	LDH	PR	IC$_{50}$ Saq (μM)	IC$_{50}$ Saq-NO (μM)
F/2	ALL	IR	35.7	90	4740	Good	49	30
M/4	ALL	IR	70.2	94	2676	Good	127	43
M/15	ALL T	HR	77.2	96	10,120	Poor	84	31
M/10	ALL	IR	7.65	37	1882	Good	37	30.9
M/4	ALL	IR	9.75	49	924	Good	14.2	9.3
F/6	ALL	IR	2.01	67	821	Good	61	75
M/15	CML/ALL	HR	163	12	2050	Poor	34	16
M/17	ALL	IR	10.3	90	1089	Good	109	31

players in signal transduction, we performed bioinformatic analysis of homology regions between active site of HIV protease and important signaling molecules in cancer cells. This evaluation revealed that there is no homology with Akt, ERK1/2, p70S6K2 and S6 protein (Suppl. 1). However, certain homologies were found within autoinhibitory domain of p70S6K1 (Suppl. 2).

Since multiple phosphorylations at the autoinhibitory domain are required, the possible influence of Saq or Saq-NO on p70S6K function was analyzed by Western blot. Analysis was performed in Jurkat and HL-60 cells at indicated time points after exposure to Saq or Saq-NO. A strong and persistent inhibition of Thr421/Ser424 phosphorylation was detected after treatment with Saq-NO in both cell lines (Fig. 3). In parallel, a strong but reversible, abrogation of S6K phosphorylation at position Thr389 was observed upon the treatment with original compound in both tested cell lines. Finally, phosphorylation of downstream target of S6K, S6 protein, was noticeably reduced by Saq-NO in both – Jurkat and HL-60 cells. As compared to Saq-NO, Saq selectively downregulated S6 activity in HL-60 but not in Jurkat cells. However, after normalization to the house-keeping protein – tubulin, this effect disappears (Suppl. 3). This is the consequence of enhancement of total amount of S6 protein. Given the observation time for the experiment, this effect is likely due to increased S6 mRNA stability rather than de novo transcription.

3.5. Modified HIV protease inhibitors are promising candidates for the treatment of hematological malignancies

To determine whether the anticancer properties of Saq-NO were unique, the HIV protease inhibitor Lopinavir was modified by attachment of a NO moiety. Jurkat, Raji and K562 cell lines were treated with various concentrations of the parental and NO-modified drugs for 48 h and then the IC$_{50}$ values were determined (Table 3). While both drugs exhibited anticancer potential, the effects of Lopinavir-NO were less potent than those previously observed for Saq-NO. For estimation of the IC$_{50}$ values of Lopinavir-NO, the effects of equimolar concentrations of Lopinavir and Lopinavir-NO were examined. Strong inhibition of cellular proliferation was observed upon the treatment with modified drug (Table 3). Lopinavir-NO induced caspase dependent apoptosis (Table 3). In summary, these data indicated that NO-derived HIV

protease inhibitors may represent promising candidate for blood cancer treatment.

4. Discussion

Covalent attachment of NO to the first approved HIV protease inhibitor Saq induces numerous advantages to original drug. While the novel compound retained the antiviral potential of parental drug, its efficacy against different cancer cell lines, as well as in syngeneic and xenograft solid tumor models was markedly enhanced [20–25]. Saq-NO also appeared to be significantly less toxic than Saq both in vitro and in vivo. Thus a new chemical entity endowed with higher anticancer potential and lower toxicity than the parental drug was generated by hybridization with NO. [20,25]. The lower toxicity of Saq-NO could be at least partly ascribed to its cytostatic activity based on inhibited proliferation followed by differentiation toward more mature phenotype as opposed to cancer cell apoptosis [20,25]. This type of antitumor action eliminates compensatory proliferation as a response of anaplastic cells to the death of cells in neighborhood [32]. At the molecular level, general loss of Saq toxicity could be associated with the fact that Saq-NO does not compromise Akt function, that might be responsible for numerous side effects observed upon the treatment with original compound such as development of diabetes, and osteoporosis [3]. Although the inhibition of Akt by Saq was linked to its antitumor potential, the NO-modified form of the drug possesses even higher capacity to suppress tumor progression without Akt down-regulation [20,25]. Recent studies elaborated the possibility that the leading target of Saq-NO belongs to downstream part of the PI3K-Akt signaling pathway, focusing the attention to S6 protein inhibition [20,25].

The results presented here extend the list of tumor cells examined for the effects of Saq-NO to hematological cancers. Raji and Jurkat cell lines were both developed from pediatric patients. Saq-NO suppressed proliferation in both cell lines but neither underwent to apoptosis. The iNOS$^+$ K562 cells were developed from an adult patient with chronic myelogenous leukemia. The p53 null HL-60 cell line resembles acute promyelocytic leukemia cell. Saq-NO suppressed proliferation and induced caspase dependent apoptosis in both K562 and HL-60 cells. The different mechanism of action exerted by Saq-NO in Jurkat/Raji vs K562/HL-60 cells is in concordance with the previously observed feature of Saq-NO to inhibit the proliferation without induction of death in numerous

Table 2
Characteristics of AML patients and effects of Saq and Saq-NO on isolated PBMC.

Sex/age	FAB AML type	WBC × 10^9/l	Blasts (%)	sLDH (U/l)	IC$_{50}$ Saq (μM)	IC$_{50}$ Saq-NO (μM)
F/60	M2	81.6	25	1642	48.2	5.8
F/56	M5	109.7	73	3316	51.9	7.9
M/77	M2	21.8	5	264	62.8	28.6
F/87	M4	65	90	458	1.2	0.9
F/71	M2	124	23	2673	54.0	27.0
F/61	M2	14.5	50	678	25.7	16.1
F/80	M2	84	38	2471	61.6	17.9
F/90	M4	70	21	682	79.7	37.3

FAB, French–American–British classification; WBC, white blood count; LDH, lactate dehydrogenase (normal range 160–410 U/l); AraC, arabinoside cytosine.
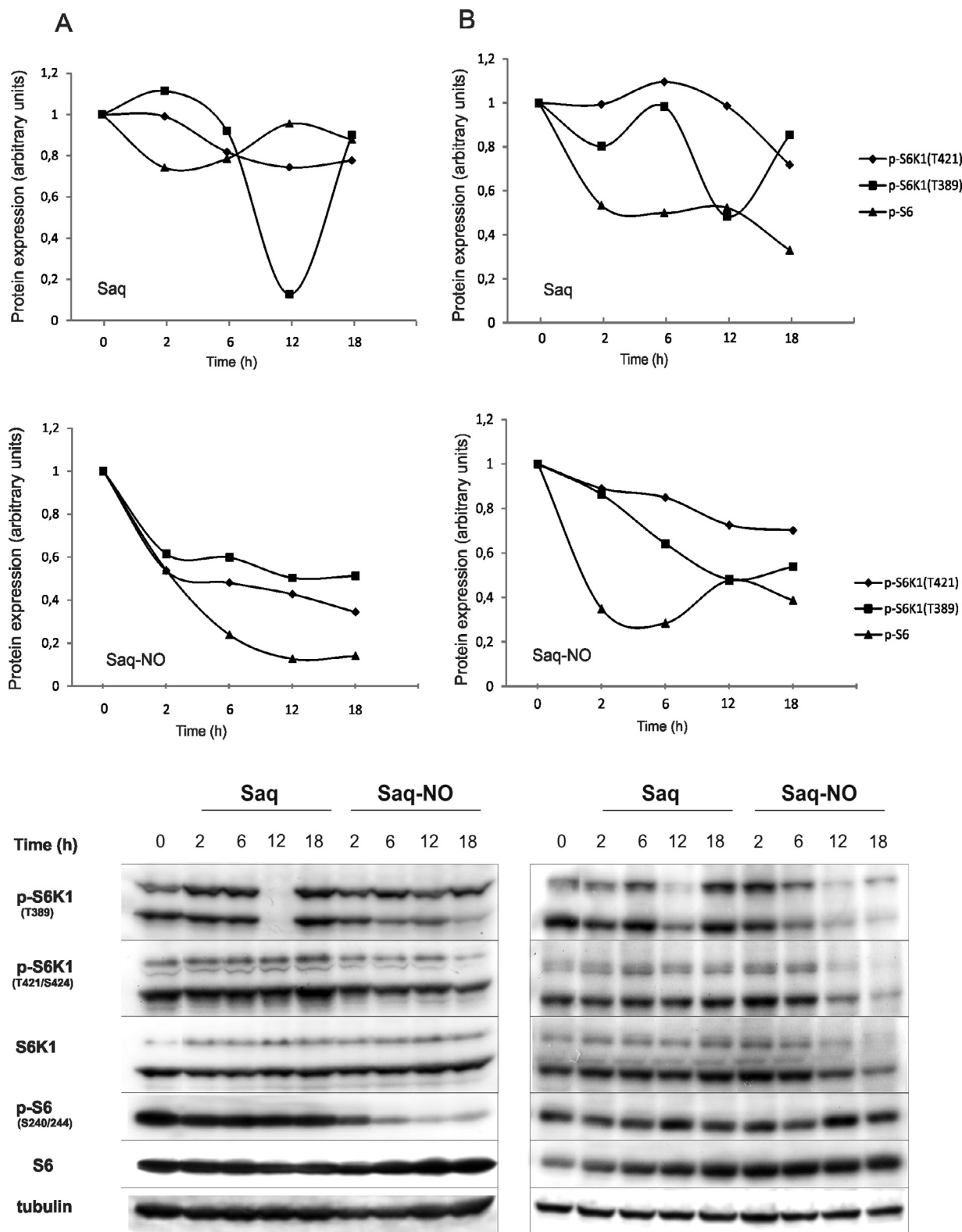
Fig. 3. Saq-NO inhibited the expression of p70S6K. Jurkat (A) and HL-60 (B) cells were exposed to IC$_{50}$ dose of Saq-NO and equal dose of Saq and expression of p70S6K and S6 protein were analyzed by Western blot. Representative data from one of three independent experiments are presented.

cancer cell lines with exception of p53 mutant or iNOS positive cells [21,22]. The fact that Saq-NO promoted intensive apoptosis in cell lines originated from adults but not from pediatric patients, could be explained by general difference in pathogenesis between pediatric and adult blood cancers. In addition, the potential of Saq-NO was presently confirmed in experiments performed

with human material isolated from the pediatric patients with ALL which is the most frequent malignant disease in childhood, accounting 25% of all malignant disease in pediatric patients. In fact, evaluation of blood samples obtained from adult AML patients revealed good responses to the treatment with Saq-NO, although at higher concentrations than those found effective in other cancer

Table 3
Antitumor potential of Lopinavir-NO.

Cell line	Lopi				Lopi-NO				Control		
	IC_{50}	SubG	% of apop.	casp act.	IC_{50}	SubG	% of apop.	casp act.	SubG	% of apop.	casp act.
Raji	18	0.65	2.3	106.33	10	9.31	43.4	124.52	0.7	3	72.5
Jurkat	33	1.01	5.7	92	14	10.18	18	122	1.52	4.7	73.7
K562	36.5	1.1	5.2	23.9	18.5	18.7	19.1	32.2	0.6	1	22.2

cell lines or cells from adult patients with blood cancer. The fact that higher IC_{50} values were observed on patients' blood cells than in cancer cell lines may be the consequence of the presence of heterogeneous cellular population in tested samples that are collectively referred as mononuclear cells but with a percentage of malignant clones that varied from sample to sample. Because of that we believe that results obtained on cell lines used as a model for these blood cancers and that show significantly lower IC_{50} values than those observed in blood patients are much appropriate for comparison and possibly more predictable for translation of in vitro findings to the clinical setting. The high IC_{50} necessary to exert antitumoral effects on blood cells from human patients may raise a concern about the possible utilization of Saq and Saq-NO to treat leukemia. In fact, the plasma concentrations of Saq vary according to the dose administrated, but values reported do not generally exceed 10 μM. Although this might make it difficult to consider a role for Saq in the treatment of these conditions, we do not believe that this jeopardizes the possible use of Saq-NO in this setting. In fact, the lower toxicity of Saq-NO that we have previously demonstrated in vitro and in vivo [20] may allow the use of doses higher than the parental compound which may achieve plasma levels higher than 10 μM. In addition, we have previously demonstrated that Saq-NO, unlike Saq, exerts synergistic potential with other commonly used chemotherapeutic drugs [22–24].

The therapeutic potential of Saq-NO in AML is amplified by the fact that this disease is often initially insensitive to chemotherapy [31]. The excellent response to Saq-NO treatment observed in primary AML cells is very promising especially in light of the fact that patients whose blood cells were treated with Saq-NO, developed early resistance to subsequent chemotherapy (not shown). Resistance to apoptosis is one of the hallmarks of adult blood cancer in both chronic and acute form [31]. Therapy based on induction of apoptosis is usually ineffective. In contrast, pediatric ALL patients are often successfully treated with cytostatic drugs, indicating that malignant hyper-proliferation is not accompanied with compromised sensitivity to apoptosis but rather connected with inadequate development of autoregulatory mechanisms involved in limitation of immune responses [32]. Saq-NO was able to limit proliferation in both, pediatric and adults clones. However, in cell lines generated from adults, the compound promoted apoptosis.

Analysis of the molecular mechanisms of drug action revealed the role of p70S6K and its downstream targets. It was recently found that targeting of mTOR signaling pathway represents a promising approach in treatment of different hematologic malignancies [33–35]. The over-expression and/or enhanced activation of proteins involved in the initiation of translation have been shown to induce a malignant phenotype [36]. As p70S6K is a key molecule in this cascade, its inhibition by specific small molecules became of great therapeutic interest for protein synthesis blockage and subsequent decrease in cell proliferation [37]. Recent data, as well as data presented in this study, clearly indicated the powerful affinity of Saq-NO to block malignant cell division through high binding affinity to a domain of this specific kinase, preventing its activation. It was previously demonstrated that ability of Saq-NO to inhibit androgen-dependent prostate cancer

proliferation was not enhanced by further addition of the mTORC1 blocker rapamycin [25]. This, also lends indirect support to the concept that Saq-NO abrogated p70S6K activation through phosphorylation of auto-inhibitory domain and prevented subsequent conformational changes of the enzyme, necessary for its complete activation. Analysis of homology between HIV protease active site and important actors of main signaling pathways revealed presence of homology sequences in autoinhibitory domain of p70S6K1, but not in Akt, S6, p70S6K2 and ERK1/2. Saq-NO remarkably inhibited multiple phosphorylations of p70S6K auto-inhibitory and linker domains in both cell lines – Jurkat and HL60. This, further, reflected on its downstream activity. The ability of Saq-NO to abolish apoptotic resistant phenotype and sensitize cells to apoptosis triggered by chemotherapy as well as immune system mediated cytotoxicity previously described in melanoma, androgen dependent and independent prostate cancers as well as non-small lung cancer, could be explained by its ability to compromise S6K dependent processes [21,23,24]. Intracellular sensitization to apoptosis is mainly associated to abrogated level of natural caspase inhibitors, which belongs to the group of proteins regulated by p70S6K. In addition, Saq-NO can enhance the expression of functional membrane death receptor specific for TRAIL-DR as well as inhibit the activity of multidrug resistant pumps (MDRs) more potently than the commercial MDR inhibitor Dexverapamil [26].

Permanent blockage of this important kinase was observed in both Jurkat and HL60 cell line and has numerous repercussions on the behavior of proliferative cells. However, in resting, non-proliferative cells, p70S6K1 is associated with eIF3 complex which centrally regulates protein synthesis [38]. Therefore Saq-NO-induced inhibition of eIF3 activity explains why Saq-NO is basically an antiproliferative drug with limited toxicity toward non-proliferative cells. It is evident that Saq-NO remarkably inhibited p70S6K phosphorylation in both Jurkat and HL60 cell lines. However, on the contrary to the expected inhibition of the downstream S6 protein observed in Jurkat cells, an increase in its phosphorylation levels was observed in HL-60 upon normalization to tubulin. This effect is due to an unexpected upregulation of total S6 protein. It was previously shown that commitment of HL-60 cells to proliferate or differentiate into monocytic/macrophage or granulocytic linage is defined by mRNA stability for ribosomal proteins [39]. Elevated phosphorylation levels of S6 in HL-60 cells have been already described by Couldwel et al. in connection with PKC depletion [40]. Interestingly, impressive similarities were found by bioinformatics analysis between HIV protease active site with the members of PKC family especially with epsilon isoform (Suppl. 1). This discovery brings a new light on our understanding of Saq/Saq-NO intracellular activities. In parallel with this, PKC inhibition is tightly related to induction of apoptosis [40]. Connection between PKC inhibition, S6 activation and apoptosis indicated the possibility that PKC, besides S6K1 was affected by Saq-NO. This interaction as well as its consequences will be the subject of future study.

Finally, Saq is the first approved HIV inhibitor and first NO-modified compound in a group of HIV protease inhibitors with demonstrated antitumoral potential. The observations that Lopinavir and importantly the enhanced ability of NO-modified Lopinavir to exert significant anti-cancer activities against blood cancer cells, points out the significant novel potential of this group

of antiviral drugs and of the related compounds obtained via the addition of NO moieties, to the field of oncotherapy.

Conflict of interest

Ferdinando Nicoletti is co-founder and shareholder of OncoNox. None of the other authors declares conflicts of interest.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2015.06.013

References

[1] J.A. McCubrey, L.S. Steelman, S.L. Abrams, N. Misaghian, W.H. Chappell, J. Basecke, et al., Targeting the cancer initiating cell: the ultimate target for cancer therapy, Curr. Pharm. Des. 18 (13) (2012) 1784–1795.

[2] J.A. McCubrey, L.S. Steelman, W.H. Chappell, S.L. Abrams, R.A. Franklin, G. Montalto, et al., Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR cascade inhibitors: how mutations can result in therapy resistance and how to overcome resistance, Oncotarget 3 (10) (2012) 1068–1111.

[3] C. Sgadari, P. Monini, G. Barillari, B. Ensoli, Use of HIV protease inhibitors to block Kaposi's sarcoma and tumour growth, Lancet Oncol. 4 (9) (2003) 537–547.

[4] G. Barillari, A. Iovane, I. Bacigalupo, C. Palladino, S. Bellino, P. Leone, et al., Ritonavir or saquinavir impairs the invasion of cervical intraepithelial neoplasia cells via a reduction of MMP expression and activity, AIDS 26 (8) (2012) 909–919.

[5] F. Timeus, N. Crescenzio, A. Doria, L. Foglia, S. Pagliano, E. Ricotti, et al., L. Cordero di Montezemolo, In vitro anti-neuroblastoma activity of saquinavir and its association with imatinib, Oncol. Rep. 27 (3) (2012) 734–740.

[6] F. Timeus, N. Crescenzio, E. Ricotti, A. Doria, D. Bertin, G. Saglio, P.A. Tovo, The effects of saquinavir on imatinib-resistant chronic myelogenous leukemia cell lines, Haematologica 91 (5) (2006) 711–712.

[7] E. Toschi, C. Sgadari, L. Malavasi, I. Bacigalupo, C. Chiozzini, D. Carlei, et al., Human immunodeficiency virus protease inhibitors reduce the growth of human tumors via a proteasome-independent block of angiogenesis and matrix metalloproteinases, Int. J. Cancer 128 (1) (2011) 82–93.

[8] K. McLean, N.A. Van De Ven, D.R. Sorenson, S. Daudi, J.R. Liu, The HIV protease inhibitor saquinavir induces endoplasmic reticulum stress, autophagy, and apoptosis in ovarian cancer cells, Gynecol. Oncol. 112 (3) (2009) 623–630.

[9] J.J. Gills, J. Lopiccolo, J. Tsurutani, R.H. Shoemaker, C.J. Best, M.S. Abu-Asab, et al., A lead HIV protease inhibitor, is a broad-spectrum anticancer agent that induces endoplasmic reticulum stress, autophagy, and apoptosis in vitro and in vivo, Clin. Cancer Res. 13 (17) (2007) 5183–5194.

[10] K.C. Cuneo, T. Tu, L. Geng, A. Fu, D.E. Hallahan, C.D. Willey, HIV protease inhibitors enhance the efficacy of irradiation, Cancer Res. 67 (10) (2007) 4886–4893.

[11] W.A. Chow, C. Jiang, M. Guan, Anti-HIV drugs for cancer therapeutics: back to the future? Lancet Oncol. 10 (2009) 61–71.

[12] M. Kraus, J. Bader, H. Overkleeft, C. Driessen, Nelfinavir augments proteasome inhibition by bortezomib in myeloma cells and overcomes bortezomib and carfilzomib resistance, Blood Cancer J 3 (2013) e103.

[13] M. Kraus, H. Müller-Ide, T. Rückrich, J. Bader, H. Overkleeft, C. Driessen, 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, Leuk. Res. 38 (3) (2014) 383–392.

[14] T. Ikezoe, E.S. Daar, J. Hisatake, H. Taguchi, H.P. Koeffler, HIV-1 protease inhibitors decrease proliferation and induce differentiation of human myelocytic leukemia cells, Blood 96 (10) (2000) 3553–3559.

[15] T. Ikezoe, T. Saito, K. Bandobashi, Y. Yang, H.P. Koeffler, H. Taguchi, HIV-1 protease inhibitor induces growth arrest and apoptosis of human multiple myeloma cells via inactivation of signal transducer and activator of transcription 3 and extracellular signal-regulated kinase 1/2, Mol. Cancer Ther. 3 (4) (2004) 473–479.

[16] C. Bono, L. Karlin, S. Harel, E. Mouly, S. Labaume, L. Galicier, et al., The human immunodeficiency virus-1 protease inhibitor nelfinavir impairs proteasome

[17] T. Ikezoe, K. Bandobashi, Y. Yang, S. Takeuchi, N. Sekiguchi, S. Sakai, et al., HIV-1 protease inhibitor ritonavir potentiates the effect of 1,25-dihydroxyvitamin D3 to induce growth arrest and differentiation of human myeloid leukemia cells via down-regulation of CYP24, Leuk. Res. 30 (8) (2006) 1005–1011.

[18] A. Beloqui, Solinís MÁ, A.R. Gascón, A. del Pozo-Rodríguez, A. des Rieux, V. Préat, Mechanism of transport of saquinavir-loaded nanostructured lipid carriers across the intestinal barrier, J. Control. Release 166 (2) (2013) 115–123.

[19] B. Rigas, J.L. Williams, NO-donating NSAIDs and cancer: an overview with a note on whether NO is required for their action, Nitric Oxide 19 (2008) 199–204.

[20] D. Maksimovic-Ivanic, S. Mijatovic, D. Miljkovic, L. Harhaji-Trajkovic, G. Timotijevic, M. Mojic, et al., The antitumor properties of a nontoxic, nitric oxide-modified version of saquinavir are independent of Akt, Mol. Cancer Ther. 8 (5) (2009) 1169–1178.

[21] S. Mijatovic, D. Maksimovic-Ivanic, M. Mojic, G. Timotijevic, D. Miljkovic, K. Mangano, et al., Cytotoxic and immune-sensitizing properties of nitric oxide-modified Saquinavir in iNOS-positive human melanoma cells, J. Cell. Physiol. 226 (7) (2011) 1803–1812.

[22] M. Donia, D. Maksimovic-Ivanic, S. Mijatovic, M. Mojic, D. Miljkovic, G. Timotijevic, et al., In vitro and in vivo anticancer action of Saquinavir-NO, a novel nitric oxide-derivative of the protease inhibitor saquinavir, on hormone resistant prostate cancer cells, Cell Cycle 10 (February (3)) (2011) 492–499.

[23] M. Donia, K. Mangano, P. Fagone, R. De Pasquale, F. Dinotta, M. Coco, et al., Unique antineoplastic profile of Saquinavir-NO, a novel NO-derivative of the protease inhibitor Saquinavir, on the in vitro and in vivo tumor formation of A375 human melanoma cells, Oncol. Rep. 28 (2) (2012) 682–688.

[24] M. Mojic, D. Maksimovic-Ivanic, S. Dinic, N. Grdovic, D. Miljkovic, et al., Saquinavir-NO-targeted S6 protein mediates sensitivity of androgen-dependent prostate cancer cells to TRAIL, Cell Cycle 11 (6) (2012) 1174–1182.

[25] M. Mojic, S. Mijatovic, D. Maksimovic-Ivanic, D. Miljkovic, S. Stosic-Grujicic, M. Stankovic, et al., Therapeutic potential of nitric oxide-modified drugs in colon cancer cells, Mol. Pharmacol. 82 (4) (2012) 700–710.

[26] F. Rothweiler, M. Michaelis, P. Brauer, J. Otte, K. Weber, B. Fehse, et al., Anticancer effects of the nitric oxide-modified saquinavir derivative saquinavir-NO against multidrug-resistant cancer cells, Neoplasia 12 (12) (2010) 1023–1030.

[27] D. Maksimovic-Ivanic, S. Stosic-Grujicic, F. Nicoletti, S. Mijatovic, Resistance to TRAIL and how to surmount it, Immunol. Res. 52 (1–2) (2012) 157–168.

[28] A. Savic, M. Dulovic, J.M. Poljarevic, S. Misirlic-Dencic, M. Jovanovic, A. Bogdanovic, et al., Synthesis and in vitro anticancer activity of ruthenium-cymene complexes with cyclohexyl-functionalized ethylenediamine-N,N′-diacetate-type ligands, Chem. Med. Chem. 6 (10) (2011) 1884–1891.

[29] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.

[30] S.F. Altschul, J.C. Wootton, E.M. Gertz, R. Richa Agarwala, A. Morgulis, A.A. Schäffer, Y. Yu, Protein database searches using compositionally adjusted substitution matrices, FEBS J. 272 (2005) 5101–5109.

[31] U. Testa, R. Riccioni, Deregulation of apoptosis in acute myeloid leukemia, Haematologica 92 (1) (2007) 81–94.

[32] R.B. Richardson, Promotional etiology for common childhood acute lymphoblastic leukemia: the infective lymphoid recovery hypothesis, Leuk. Res. 35 (11) (2011) 1425–1431.

[33] N. Chapuis, J. Tamburini, A.S. Green, L. Willems, V. Bardet, S. Park, et al., Perspectives on inhibiting mTOR as a future treatment strategy for hematological malignancies, Leukemia 24 (10) (2010) 1686–1699.

[34] A. Younes, N. Samad, Utility of mTOR inhibition in hematologic malignancies, Oncologist 16 (6) (2011) 730–741.

[35] P. Fagone, M. Donia, K. Mangano, C. Quattrocchi, S. Mammana, M. Coco, et al., Comparative study of rapamycin and temsirolimus demonstrates superimposable anti-tumour potency on prostate cancer cells, Basic Clin. Pharmacol. Toxicol. 112 (1) (2013) 63–69.

[36] P.R. Hagner, A. Schneider, R.B. Gartenhaus, Targeting the translational machinery as a novel treatment strategy for hematologic malignancies, Blood 115 (11) (2010) 2127–2135.

[37] L.R. Pearce, G.R. Alton, D.T. Richter, J.C. Kath, L. Lingardo, J. Chapman, et al., Characterization of PF-4708671, a novel and highly specific inhibitor of p70 ribosomal S6 kinase (S6K1), Biochem. J. 431 (2) (2010) 245–255.

[38] T.R. Fenton, I.T. Gout, Functions and regulation of the 70 kDa ribosomal S6 kinases, Int. J. Biochem. Cell Biol. 43 (1) (2011) 47–59.

[39] A. Grolleau, N. Sonenberg, J. Wietzerbin, L. Beretta, Differential regulation of 4E-BP1 and 4E-BP2, two repressors of translation initiation, during human myeloid cell differentiation, J. Immunol. 162 (6) (1999) 3491–3497.

[40] W.T. Couldwell, D.R. Hinton, S. He, T.C. Chen, I. Sebat, M.H. Weiss, R.E. Law, Protein kinase C inhibitors induce apoptosis in human malignant glioma cell lines, FEBS Lett. 45 (1) (1994) 43–46.