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Antibody-targeted horseradish peroxidase associated with indole-3-acetic acid induces apoptosis *in vitro* in hematological malignancies

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ABSTRACT

Indole-3-acetic acid (IAA), when oxidized by horseradish peroxidase (HRP), is transformed into cytotoxic molecules capable of inducing cell injury. The aim of this study was to test if, by targeting hematopoietic tumors with HRP-conjugated antibodies in association with IAA treatment, there is induction of apoptosis. We used two lineages of hematologic tumors: NB4, derived from acute promyelocytic leukemia (APL) and Granta-519 from mantle cell lymphoma (MCL). We also tested cells from 12 patients with acute myeloid leukemia (AML) and from 10 patients with chronic lymphocytic leukemia (CLL). HRP targeting was performed with anti-CD33 or anti-CD19 antibodies (depending on the origin of the cell), followed by incubation with goat anti-mouse antibody conjugated with HRP. Eight experimental groups were analyzed: control, HRP targeted, HRP targeted and incubated with 1, 5 and 10 mM IAA, and cells not HRP targeted but incubated with 1, 5 and 10 mM IAA. Apoptosis was analyzed by flow cytometry using annexin V-FITC and propidium iodide labeling. Results showed that apoptosis was dependent on the dose of IAA utilized, the duration of exposure to the prodrug and the origin of the neoplasia. Targeting HRP with antibodies was efficient in activating IAA and inducing apoptosis.

1. Introduction

One of the goals of antitumor therapy is to selectively target toxic agents to tumor cells, sparing normal tissues of injury, which can be achieved by antibodies that recognize specific markers on the tumor cell surface. In this regard, enzymes capable of activating prodrugs to active drugs have been the focus of considerable recent interest, and conjugation of the enzyme to an antibody could allow it to be targeted selectively to the tumor after intravenous administration, a procedure called antibody-directed enzyme prodrug therapy (ADEPT) [1].

Indole-3-acetic acid (IAA), when oxidized by horseradish peroxidase (HRP), produces toxic species that could be used as the basis for a novel anticancer therapy [2,3]. IAA is an important plant growth phytohormone that plays a role in the regulation of cell division, elongation and differentiation [4]. It has been detected in human urine, blood, cerebrospinal fluid and several other organs [5,6] and is obtained from a diet rich in vegetable stems or synthesized from tryptophan [7]. IAA appears to be well tolerated in humans [8] and mice [9], although myotonia and hypothermia were observed in mice at the dose of 300 mg/kg [9]. HRP is a heme-containing peroxidase enzyme that can oxidize a wide variety of substrates in the presence of hydrogen peroxide [10]. It has been reported that HRP can oxidize IAA even in the absence of hydrogen peroxide, leading to toxicity to mammalian cells in conditions of either normoxia or anoxia [11].

The mechanism of toxicity towards cells is extremely complex and not fully understood. It has been reported that IAA activated by HRP produces free radicals such as indolyl, skatolyl and peroxyl radicals, which can cause membrane lipid peroxidation [2,12]. Effects on the nuclear compartment of the cell and on the DNA were also reported [2]. Both membrane and nuclear injuries were prevented when cells were treated with antioxidants, such as ascorbate, vitamin E, and β-carotene [13,14]. Incubation of rat neutrophils but not of lymphocytes with IAA induced cell death, demonstrated by loss of membrane integrity, chromatin condensation and DNA fragmentation [6], which suggests that the myeloperoxidase present in neutrophils can activate IAA as well as HRP. When HRP was added to the lymphocyte culture or the lymphocytes were co-cultivated with neutrophils and IAA, apoptosis was detected [6].

Toxicity of IAA/HRP was demonstrated in many human tumor cell lineages, such as T24 bladder carcinoma [11], MCF-7 breast adenocarcinoma [11], FaDu nasopharyngeal squamous carcinoma

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Table 1
Clinical characteristics from AML patients.

Patient	Age (years)	Sex	Hemoglobin (g/dL)	WBC ($\times 10^6$/L)	PB blasts (%)	Platelets ($\times 10^6$/L)	BM blasts (%)	FAB Classification
1	58	F	7.7	149,700	95	36,000	99	M1
2	28	F	7.2	32,600	84	10,000	75	M2
3	64	F	8.4	700	0	57,000	71	M2
4	53	M	8.6	21,700	67	86,000	71	M2
5	50	M	7.6	70,900	85	30,000	95	M4
6	62	M	9.4	100,000	98	19,000	100	M4
7	53	F	8.7	2500	24	34,000	100	M3
8	30	M	12.3	1300	0	163,000	37	M3
9	27	F	10.9	1100	16	33,000	100	M3
10	32	M	11.4	4400	8	29,000	100	M3
11	43	F	5.7	500	0	7000	95	M3
12	18	M	7.3	26,300	90	37,000	100	M3

WBC: white blood cell, PB: peripheral blood, BM: bone marrow, FAB: French-American-British.

[11], G361 melanoma cells [15], BXPC-3 pancreatic cancer [16] and a human head and neck carcinoma cell line [17].

Our aim was to evaluate apoptosis induced by IAA/HRP in myeloid and lymphoid malignancies using cell lineages and primary tumor cells from patients with acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL).

2. Material and methods

2.1. Cell lineages

We used the cell lineages NB4 and Granta-519. The first is derived from blasts of human acute promyelocytic leukemia (APL), harbors the t(15;17)(q22;q12) translocation and expresses typical immunophenotypic markers such as CD33, CD13, CD15 and cytoplasmic myeloperoxidase [18]. The Granta-519 cell lineage is derived from human mantle cell lymphoma (MCL), harboring the t(11;14)(q13;q32) translocation with overexpression of cyclin D1 [19]. These cells also express characteristic surface markers such as CD19, FMC7, CD23 and λ light chain [19]. The NB4 lineage was kindly provided by Professor Pier Paolo Pandolfi (Harvard Medical School, Boston, USA) and Granta-519 by Professor Belinda Pinto Simões (GSF-Hämatologikum, Munich, Germany). Primary human microvascular endothelial cells from lung (HMVEC-L) (Lonza, Walkersville, MD, USA) were co-cultured with Granta-519 to evaluate the effects of IAA/HRP on normal cells.

2.2. Patients

From January/2007 to December/2008, 22 patients from the University Hospital of the Medical School of Ribeirão Preto were enrolled in this study after giving written informed consent. Bone marrow was aspirated from 12 patients with AML (six with APL and six with AML other than APL) and peripheral blood was obtained from 10 patients with CLL and mononuclear cells were isolated by Ficoll Hypaque density gradient centrifugation (Sigma–Aldrich, St. Louis, MO, USA). The patients were diagnosed according to classical criteria established by the World Health Organization. The clinical and laboratory records of these patients were reviewed. Approval from the local Ethics Committee was obtained (protocol number 4342/2006).

2.3. HRP targeting

The HRP targeting to the tumor cells was performed based on indirect labeling utilizing monoclonal antibodies. First, 0.5×10^6 cells were incubated with 5 μL of murine anti-CD33 (NB4 and AML primary cells) or anti-CD19 (Granta-519 or CLL primary cells). Both antibodies were purchased from BD Pharmingen (San Diego, USA) at the concentration of 0.5 mg/mL. After washing with Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, NY, USA) as a second step, these cells were incubated with 5 μL of goat-anti-mouse antibody conjugated with HRP (GAM-HRP) (Dako, Carpinteria, CA, USA) at the concentration of 1 mg/mL. After these two steps, the cells were resuspended in 1000 μL of RPMI 1640 medium supplemented with 20% fetal calf serum and 40 mg/L gentamicin.

2.4. Cell culture

Eight experimental groups were analyzed: controls (in which no antibody was added), cells targeted with HRP, cells targeted with HRP and incubated with 1, 5 or 10 mM IAA and cells not targeted but incubated with 1, 5 or 10 mM IAA (Vetec Quimica Fina Ltd, Rio de Janeiro, Brazil). NB4 and Granta-519 cells were cultured at 5% CO_2/37 °C in quintuplicate and tested for apoptosis after 2, 8, 18, 24 and 48 h of incubation. Similarly, cells from AML and CLL patients were divided into 16 aliquots and cultured under the same conditions for 2 and 24 h. IAA was dissolved in 10% ethanol, and controls were treated with the same vehicle. The final ethanol concentration in each well was 1%.

Separately, HMVEC-L cells were grown in EGM-2MV medium (Lonza, Walkersville, MD, USA) supplemented as indicated by the manufacturer. A total of 0.5×10^6 Granta-519 cells were added to this culture after HMVEC-L cells reached 90% confluence (around 0.5×10^5 cells). Six experimental groups were incubated for 24 h and then analyzed: controls (in which no antibody was added), HMVEC-L cells with HRP-targeted Granta-519 cells, HMVEC-L cells with HRP-targeted Granta-519 cells treated with 1 or 5 mM IAA, HMVEC-L cells with Granta-519 cells (not HRP targeted) treated with 1 or 5 mM IAA. The HRP targeting to Granta-519 cells was performed before they were added to HMVEC-L cells culture. After 24 h, the two lineages were separated: suspended Granta-519 cells were removed and adhered HMVEC-L cells were released after 10 min incubation with trypsinization reagent (Lonza, Walkersville, MD, USA). Trypsin was then neutralized with 4 mL of Trypsin Neutralizing Solution (Lonza, Walkersville, MD, USA). In this experiment, HMVEC-L cells were exposed to the IAA/HRP effects in the culture medium. Granta-519 but not HMVEC-L cells were HRP-targeted, and the effects of this tumor treatment were analyzed in the endothelial cells by evaluation of apoptosis as described below.

2.5. Apoptosis analysis

After culture, cells were washed in binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$) and incubated with FITC-conjugated annexin V (BD Pharmingen, San Diego, USA) and 0.5 μg/mL propidium iodide (PI). Flow cytometric analysis was performed on a FACScalibur (Becton Dickinson, San Jose, CA, USA), where 10,000 cells were acquired for fluorescence measurements. Annexin V-positive cells were considered to be apoptotic.

2.6. Statistical analysis

A linear mixed effect model was proposed to compare differences in percentages of apoptotic cells between groups. These models are based on the assumption that their residues have normal distribution with 0 mean and σ^2 variance. The model proposed for each variable is as follows: $y_{ijk} = \eta + \omega_i + \beta_j + \delta_k + (\beta\delta)_{jk} + \varepsilon_{ijk}$, where y_{ijk} is the observation of the response variable for the ith individual in the jth group and at the kth time ($i = 1, \ldots, 5$; $j = 1, \ldots, 8$; $k = 1, \ldots, 5$); η is a constant (an intercept); ω_i is a random effect ($i = 1, \ldots, 5$); β_j is the effect of the jth group, δ_k is the effect of the kth time, $\beta\delta_{jk}$ is the effect of group-by-time interaction, and ε_{ijk} is the associated error with $N(0, \sigma^2)$ distribution. These statistical analyses were performed using the SAS software version 9.

Dose–effect relations were calculated using values of four IAA doses (0, 1, 5 and 10 mM). CalcuSyn version 2.0 was the software used to calculate the r coefficient of the correlation between dose and effect (percentage of apoptotic cells) and the dose at which 50% of the effect was observed (apoptotic cells) (ED50). An α value ≤ 0.05 was considered to be significant.

3. Results

3.1. Patient cohort

Tables 1 and 2 summarize the clinical characteristics of the patients, and Tables 3 and 4, the immunophenotypic characteristics. AML patients (mean age: 43 years) had mean hemoglobin level of 8.7 g/dL, a mean white blood cell count of $34{,}300 \times 10^6$/L, and a mean platelet count of $45{,}000 \times 10^6$/L. Of 12 patients with AML, 6 were diagnosed as having APL. Ten patients diagnosed with CLL were enrolled. The mean age of these patients was 69 years, mean hemoglobin level was 13.2 g/dL, mean white blood cell count $60{,}700 \times 10^6$/L, mean lymphocyte count $48{,}660 \times 10^6$/L, and mean

Table 2
Clinical characteristics from CLL patients.

Patient	Age (years)	Sex	Hemoglobin (g/dL)	WBC ($\times 10^6$/L)	Lymphocytes ($\times 10^6$/L)	Platelets ($\times 10^6$/L)	Binet
1	62	M	14.8	38,600	31,800	110,000	A
2	85	M	12.3	35,500	25,200	210,000	A
3	66	M	10.5	63,100	41,200	413,000	B
4	70	M	14.6	30,600	25,800	196,000	A
5	66	F	13.3	55,300	43,700	217,000	A
6	72	M	13.9	76,300	45,800	319,000	B
7	77	M	15.4	18,6400	18,0000	103,000	B
8	70	F	10.1	76,000	60,800	202,000	A
9	79	M	13.0	19,400	14,200	141,000	B
10	49	F	13.3	26,300	18,100	162,000	A

WBC: white blood cells.

Table 3
Immunophenotypic characteristics from AML patients.

Patient	Immunophenotypic markers	
	Positive markers	Negative markers
1	CD33, CD13, HLA-DR, CD117, CD34, CD11b, MPO	CD42a, CD2
2	CD33, CD13, HLA-DR, CD117, CD34, MPO	CD42a, CD2, CD19, CD11b
3	CD33, CD13, HLA-DR, CD117, CD34, MPO	CD42a, CD2, CD19, CD11b
4	CD33, CD13, HLA-DR, CD117, CD34, CD15, MPO	CD42a, CD2, CD19, CD11b
5	CD33, CD13, HLA-DR, CD117, CD11b, MPO	CD42a, CD2, CD19, CD34
6	CD33, CD13, HLA-DR, CD117, CD11b, CD15, MPO	CD42a, CD2, CD19, CD34
7	CD33, CD13, CD117, MPO	HLA-DR, CD2, CD11b, CD34
8	CD33, CD13, CD117, CD15, MPO	HLA-DR, CD42a, CD2, CD34
9	CD33, CD13, CD117, CD34, MPO	HLA-DR, CD42a, CD2, CD15
10	CD33, CD13, CD117, CD15, MPO	HLA-DR, CD42a, CD2, CD34
11	CD33, CD13, CD117, CD15, MPO	HLA-DR, CD42a, CD2, CD34
12	CD33, CD13, CD117, CD34, MPO	HLA-DR, CD42a, CD2, CD15

Positive reaction to a given antibody is defined as a minimum threshold of 20% positive blasts to the respective antigen. MPO: myeloperoxidase.

platelet count 207,300 $\times$ 10^6/L. All patients presented at the initial stages of the disease (Binet classification A or B).

3.2. Apoptosis in NB4 lineage

Fig. 1A shows the percentage of apoptotic cells in NB4 samples treated with IAA associated or not with antibody-directed HRP. All the control groups (cells only, cells targeted with HRP and not incubated with IAA and cells incubated with IAA and not targeted with HRP) presented low percentages of apoptotic cells with no statistical differences between them. After 8 h of incubation, only cells targeted with HRP and treated with 10 mM IAA presented a significantly higher percentage of apoptotic cells compared to control groups (mean 32.2% *versus* 11.1–14.9% in the control groups, $p \leq 0.05$). After 18 h, cells targeted and treated with 5 and 10 mM IAA presented 52.3 and 32.2% rates of apoptosis, respectively (controls ranged from 10.2 to 15.7%, $p \leq 0.05$). After 24 and 48 h, all groups targeted with HRP and treated with 1, 5 and 10 mM IAA presented a significantly higher percentage of apoptotic cells compared to the control groups ($p \leq 0.05$).

3.3. Apoptosis in the Granta-519 lineage

Apoptosis induction by the different treatments in Granta-519 cells is shown in Fig. 1B. We found higher apoptosis in all cells targeted and treated with 1, 5 and 10 mM IAA starting at 8 h of culture compared to the control groups. After 8 h, cells targeted and treated with 1, 5 and 10 mM IAA presented 25.2, 31.8 and 41.3% rates apoptotic cells, respectively, while in the control groups these percentages ranged from 13.1 to 15.6% ($p \leq 0.05$). After 18, 24 and 48 h, apoptosis in targeted and treated cells reached levels of approximately 70%, while control groups presented levels between 8.9 and 20.6% ($p \leq 0.05$).

3.4. Apoptosis in the HMVEC-L lineage

The percentages of apoptotic cells in the HMVEC-L lineage that was co-cultured with Granta-519 are shown in Fig. 2. After 24 h of

Table 4
Immunophenotypic characteristics from CLL patients.

Patient	Immunophenotypic markers	
	Positive markers	Negative markers
1	CD19, CD5, CD23, κ light chain, CD38	FMC7, CD79b, CD2, CD10, λ light chain
2	CD19, CD5, CD23, λ light chain	FMC7, CD79b, CD2, CD10, κ light chain, CD38
3	CD19, CD5, CD23, λ light chain, CD38	FMC7, CD79b, CD2, CD10, κ light chain
4	CD19, CD5, CD23, CD38	FMC7, CD79b, CD2, CD10, κ and λ light chains
5	CD19, CD5, CD23, κ light chain, FMC7,	CD79b, CD2, CD10, λ light chain, CD38
6	CD19, CD5, CD23, κ light chain, CD38	FMC7, CD79b, CD2, CD10, λ light chain
7	CD19, CD5, CD23, κ light chain, CD79b	FMC7, CD2, CD10, λ light chain, CD38
8	CD19, CD5, CD23, λ light chain, CD38	FMC7, CD79b, CD2, CD10, κ light chain
9	CD19, CD5, CD23, κ light chain, CD79b	FMC7, CD2, CD10, λ light chain, CD38
10	CD19, CD5, CD23, κ light chain, CD38	FMC7, CD79b, CD2, CD10, λ light chain

Positive reaction to a given antibody is defined as a minimum threshold of 20% positive blasts to the respective antigen.

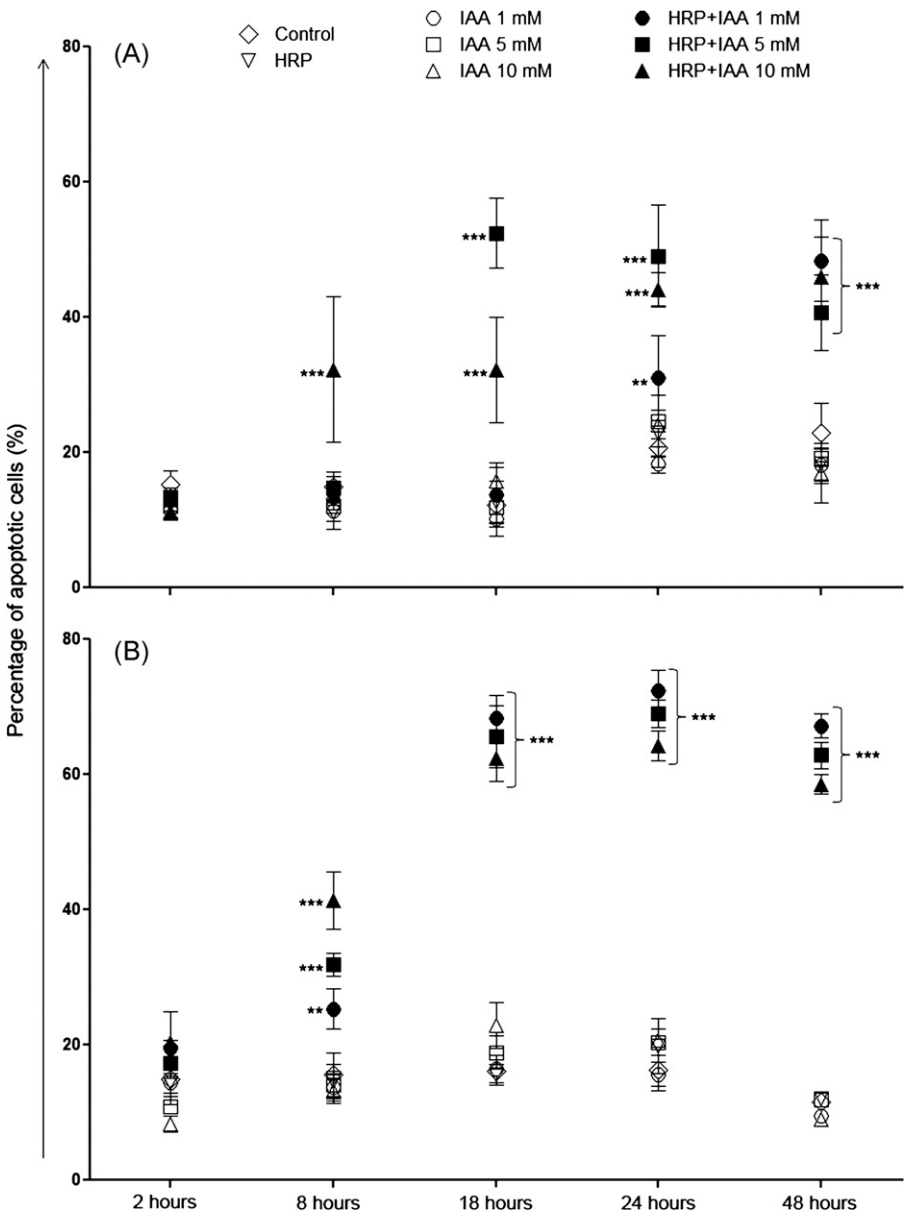

Fig. 1. Percentage of apoptotic cells according to the group of treatment and time of incubation (2, 8, 18, 24 and 48 h). (A) NB4 lineage results and (B), Granta-519 results. The apoptotic cell percentages are presented as mean ± standard error (SE). Statistical analysis is pointed according to the level of significance (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$), comparing the treated group to the control group.

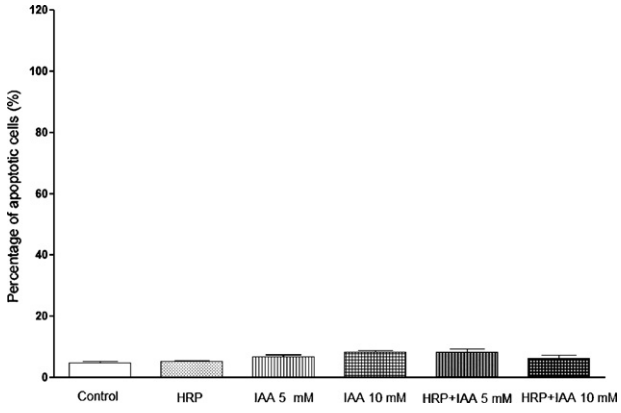

Fig. 2. Percentage of apoptosis in HMVEC-L cells that were co-cultured with Granta-519 cells, according to the group of treatment. These cells were incubated for 24 h. The bars indicate mean ± SE. No statistical significance was found between the groups.

incubation, all groups presented similar percentages of apoptosis ranging from 4.8 to 8.2%, with no significant differences.

3.5. Apoptosis in CLL patients

The mean percentages of apoptotic cells detected in CLL patients' samples are shown in Fig. 3A. After 2 h of incubation, all samples presented similar percentages of apoptosis ranging from 22.8 to 28.9%, with no statistical difference. Analysis at 24 h demonstrated that the groups targeted and treated with 1, 5 and 10 mM IAA had higher percentages of apoptotic cells (58.5, 81.3 and 93.3%, respectively) compared to the control groups (35.7–53.7%, $p \leq 0.05$) (Fig. 3A).

3.6. Apoptosis in AML patients

3.6.1. Non-APL patients

In AML other than APL, the percentage of apoptotic cells after 2 h of incubation was similar in all groups, ranging from 8.9 to 15.5%,

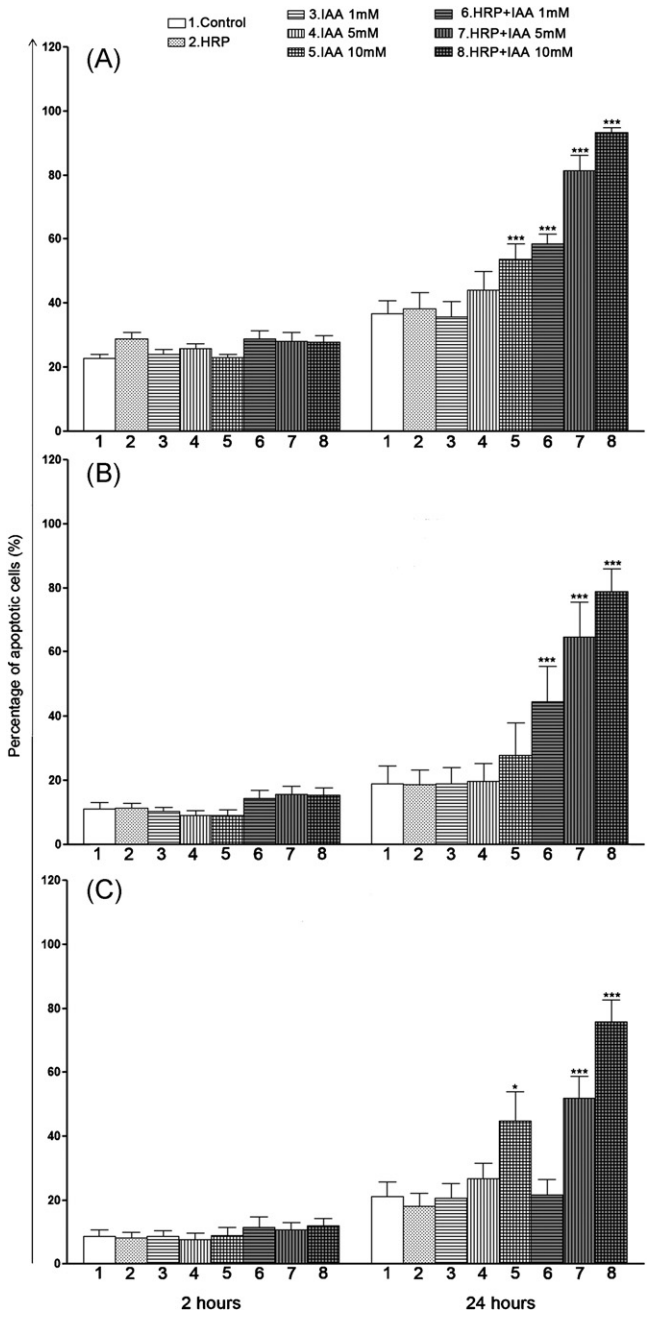

Fig. 3. Percentage of apoptotic cells according to the group of treatment and time of incubation (2 and 24 h). (A) CLL patients' results, (B) AML patients' results and (C) APL patients' results. The bars indicate mean ± SE. Statistical analysis is pointed according to the level of significance ($^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$), comparing the treated group to the control group.

with no statistical differences. In 24 h samples, targeted cells which were also treated with 1, 5 and 10 mM IAA presented higher percentages of apoptosis (44.4, 64.7 and 78.8% respectively, while in the control groups apoptosis ranged from 18.5 to 27.8%, $p \leq 0.05$) (Fig. 3B).

3.6.2. APL patients

In APL patients' samples treated for 2 h, the percentage of apoptotic cells was similar in all groups, ranging from 7.7 to 12.1%, with no statistical differences. Twenty-four hours analysis demonstrated a higher percentage of apoptotic cells only in the targeted groups treated with 5 and 10 mM IAA (51.9 and 75.9%, respectively, $p \leq 0.05$). In the control groups and in the group targeted with HRP and treated with 1 mM IAA no differences were noted (the values ranged from 18.1 to 44.8%). In these experiments, we detected that samples not targeted with HRP but treated with 10 mM IAA showed higher apoptosis induction compared to other controls (44.8%, while the other controls ranged from 18.1 to 26.8%, $p \leq 0.05$) (Fig. 3C).

Table 5
Dose in which is observed 50% of apoptosis (ED50) and coefficient *r* of correlation between dose and effect, in tumor cells treated for 24 h with IAA/HRP.

Group	ED50 (mM)	*r*
NB4	112.7	0.90
Granta-519	0.11	0.95
AML patients	0.19	0.92
APL patients	2.5	0.88
CLL patients	0.003	0.84

3.7. Dose–effect correlation

The ED50 (dose at which 50% of the cells were apoptotic) and the *r* coefficient of the correlation between dose and effect are described in Table 5 for tumor cells treated for 24 h. By comparing the Granta-519 lineage with NB4, we could demonstrate that the ED50 for the MCL lineage was about 1000 times lower than the values calculated for the NB4 lineage. Cells from CLL patients' samples also presented a much lower ED50 than that of cells from AML and APL samples. Besides, the *r* coefficient demonstrated a good dose–effect correlation in all groups, with few exceptions.

4. Discussion

Potential application of the IAA/HRP association to cancer therapy is well recognized, since it is quite evident that it can cause cell death, although toxicity mechanisms are not fully understood. The sensitivity of different cell types might not be the same [13]. Targeting the HRP enzyme to the tumor cell has already been successfully performed *in vitro* using gene therapy [11,12].

The first report of using antibodies in cancer therapy was published in 1980 [20], and, since then, many monoclonal antibodies have been developed and used successfully in the clinical treatment of hematopoietic patients [21–24]. Our results demonstrated that the antibody-directed enzyme/prodrug therapy (ADEPT) IAA/HRP could induce apoptosis in all the cells tested and, therefore, may enhance the anti-leukemia effect of monoclonal antibodies. The toxicity to Granta-519 lineage and CLL cells, both tumors of lymphoid origin, was higher compared with NB4 lineage, AML and APL cells, as demonstrated by the much higher baseline rate of apoptosis, which in turn impacts on their lower ED50 values, especially in CLL patients' samples.

The IAA doses used appear to be much higher than those previously used *in vitro* [2,3,5,11]. Probably, the HRP targeting using antibodies required these higher doses to achieve the expected toxics effects. Whether this will be possible *in vivo* remains to be evaluated.

To evaluate the fate of non-targeted normal cells, the effects of IAA/HRP on the HMVEC-L endothelial lineage was analyzed when these cells were co-cultured with HRP-targeted Granta-519 cells. The endothelial cells suffered minimal deaths, and the percentage of apoptosis was not different between them, even in the groups that were co-cultured with HRP-targeted Granta-519 cells treated with high doses of IAA. This suggests that IAA/HRP can be safe, but it is difficult to predict its toxic effects in humans. Targeting HRP to CD33 and CD19 can even harm normal granulocytic and lymphocytic cells, adding toxicity to the treatment. It is possible to

target HRP to more specific tumor markers, sparing normal cells from injury, a fact that remains to be determined.

In APL patients, the group of cells that was not HRP targeted but was treated with 10 mM IAA presented higher apoptosis when compared to the other control groups, a fact suggesting that the endogenous myeloperoxidase could alone activate IAA. Promyelocytes are cells rich in myeloperoxidase, and it has been already reported that rat neutrophils were capable of activating IAA in the absence of HRP [5,6]. Previous studies have indicated that the induction of cell death by IAA is related to the activity of peroxidase, which leads to the formation of toxic metabolites and oxygen reactive species. The APL samples that were targeted to the HRP and exposed to 10 mM IAA presented a higher percentage of apoptotic cells than those treated with IAA alone, suggesting that HRP is more effective in activating IAA than myeloperoxidase.

In summary, the association of HRP and IAA can cause apoptosis in human hematopoietic tumors which was dependent on the dose of IAA used, on the duration of exposure to the prodrug and on the origin of the neoplasia. The targeting of HRP using antibodies was effective for this purpose. To consider clinical applications, the mechanisms of toxicity must be better elucidated, the effects of IAA in humans must be carefully studied and the impact of the observed apoptosis in tumor therapy and in normal cells must be better assessed in *in vivo* models.

Conflict of interest

The authors declare that they have no potential conflicts of interest.

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Contribution: LFFD provided the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article, revised it critically for important intellectual content, and approved the final version to be submitted; BASL analyzed data, drafting of manuscript and was responsible for important intellectual content; RHJ contributed with statistical analysis; ABG performed the flow cytometry analysis; EMR and LMF analyzed data, drafting of manuscript and were responsible for important intellectual content; RPF revised the article critically for important intellectual content and gave final approval of the version to be submitted.

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