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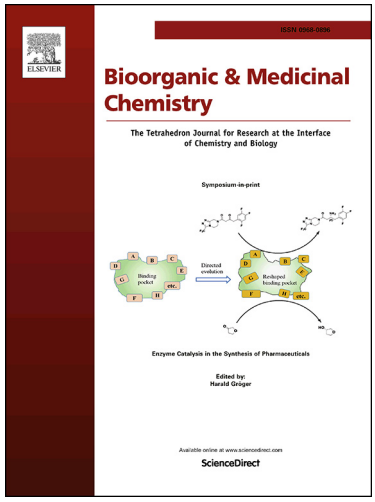

Synthesis of novel pyrazoline derivatives and the evaluation of death mechanisms involved in their antileukemic activity

Natália Marceli Stefanes, Jéssica Toigo, Mariana Franzoni Maioral, Amanda Virtuoso Jacques, Louise Domeneghini Chiaradia-Delatorre, Daiane Mari Perondi, Amanda Abdalla Biasi Ribeiro, Álisson Bigolin, Iris Mattos Santos Pirath, Bruna Ficher Duarte, Ricardo José Nunes, Maria Cláudia Santos-Silva

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SYNTHESIS OF NOVEL PYRAZOLINE DERIVATIVES AND THE EVALUATION OF DEATH MECHANISMS INVOLVED IN THEIR ANTILEUKEMIC ACTIVITY

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The authors declare no conflicts of interest regarding the publication of the present article.

Abstract

Malignant neoplasms are one of the leading causes of death worldwide and hematologic malignancies, including acute leukemia (AL) is one of the most relevant cancer types. Current available chemotherapeutics are associated with high morbidity and mortality rates, therefore, the search for new molecules with antitumor activity, specific and selective for neoplastic cells, became a great challenge for researchers in the oncology field. As pyrazolines stand out in the literature for their great variety of biological activities, the aim of this study was to synthesize and evaluate the antileukemic activity of five new pyrazoline derivatives. All pyrazolines showed adequate physicochemical properties for a good oral bioavailability. The two unpublished and most effective pyrazoline derivatives have been selected for further experiments. These compounds are highly selective for leukemic cells when compared to non-neoplastic cells and did not cause lysis on human red blood cells. Additionally, selected pyrazolines induced cell cycle arrest at G0/G1 phase and decreased cell proliferation marker KI67. Apoptotic cell death induced by selected pyrazolines was confirmed by morphological analysis, assessment of phosphatidylserine residue exposure and DNA fragmentation. Several factors indicate that both intrinsic and extrinsic apoptosis occurred. These were: increased FasR expression; the predominance of Bax in relation to Bcl-2; the loss of mitochondrial membrane potential; AIF release; decreased expression of survivin (an antiapoptotic protein); and the activation of caspase-3. The selected pyrazolines were also found to be cytotoxic against neoplastic cells collected from the peripheral blood and bone marrow of patients with different subtypes of acute leukemia.

Keywords: pyrazolines, acute leukemia, cytotoxicity, apoptosis.

1 Introduction

The isolation of natural compounds as potential chemotherapeutic drugs is limited by their low-yield, as well as by the scarcity of their natural sources [1, 2]. In addition, some natural compounds are highly cytotoxic to non-neoplastic cells; thus, although presenting antitumor properties, their therapeutic use is limited [3]. For this reason, synthetic heterocyclic compounds are of great importance due to their wide applicability in many fields, including medicinal chemistry. Pyrazolines are important examples of biologically active molecules with a five membered ring containing two atoms of nitrogen and a double bond [4, 5]. Among the several biological properties of pyrazoline derivatives are the antimicrobial, antioxidant, anti-inflammatory and antitumor activities [5], including anticancer potential in cervix carcinoma (HeLa), and lung cancer (NCI-H522), among other cell lines [6, 7].

Hematologic neoplasms are the most frequent cancer type in children, adolescents and young adults, and also present a high incidence in patients over 60 years of age [8, 9]. Acute leukemias (ALs) are a group of hematologic malignancies characterized by a blockage of normal differentiation and by the exacerbated clonal proliferation of a single myeloid or lymphoid progenitor [10]. Chemotherapy with cytotoxic and cytostatic drugs is the first therapeutic option against AL; however, it has several limitations, such as severe adverse effects and high relapse rates [6, 11]. In this perspective, since cancer is a major public health problem, the search for novel molecules that may be used as prototypes for the development of new drugs has motivated scientific researches in the oncology field [6, 12]. Thus, aiming to contribute to the discovery of safer and more effective compounds against AL, this study developed a series of pyrazoline derivatives with cytotoxic effects on leukemic cells that induce regulated cell death without significant cytotoxicity to non-neoplastic cells.

2 Material and methods

2.1 Chemistry

2.1.1 Synthesis

Reagents used were purchased from Sigma-Aldrich® or Merck®. Chalcones were obtained by aldol condensation of acetophenones (5.0 mmol) with aldehydes (5.0 mmol) in ethanol (25 ml) and KOH (50% v/v), at room temperature and magnetic stirring for 24

h,[13] and neutralized with HCl (10%v/v). The precipitate was separated by vacuum filtration and the product was recrystallized to give an average 80% yield of chalcones [3]. To the obtained chalcones (1.0 mml), acetic acid (20 mL) and hydrazine hydrate (0.2 mL, 3.0 mmol) was added, the mixture was refluxed under stirring for 6 h, and then poured in ice bath and neutralized with $NaHCO_3$(aq)[14]. The formed precipitate was filtered, washed with cold water, and recrystallized in ethanol/EtOAc (80:20v/v) to give the (3,4,5-trimethoxyphenyl-4,5-dihydro-1H-pyrazole-1-yl) ethanone derivatives. The new five pyrazoline derivatives were characterized by the melting points (m.p.), ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) and high-resolution mass spectra (HRMS) and the physicochemical data on synthesized compounds are described in Supp.Mat.1.

2.1.2 Physicochemical properties

Compounds were evaluated according to physicochemical parameters proposed by Lipinski et al. (2001)[15] and Veber et al. (2002)[16]. Molecular weight (MW), cLog P hydrogen bonding acceptors, hydrogen bonding donors, number of rotatable bonds and polar surface area (PSA) were obtained from Osiris® (Acetylion Pharmaceuticals Ltd., available at: *http://www.organic-chemistry.org*).

2.2 Biological evaluation

All experiments with human samples were approved by the Research Ethics Committee with Human Beings (CEPSH-UFSC nº746.486/2014) and all individuals signed the Free and Informed Consent Form present in the approved research project.

2.2.1 Cells

Acute myeloid leukemia (AML) (K562) and T-cell acute lymphoblastic leukemia (T-ALL) (Jurkat) cell lines were originally purchased from the American Type Culture Collection (ATCC). Murine melanoma (B16-F10) and Burkitt lymphoma (BL) (Daudi) cells were purchased from ATCC or the Rio de Janeiro Cell Bank (BCRJ). Peripheral blood mononuclear cells (PBMC) and red blood cells (RBC) were isolated from six non-smoking healthy volunteers by Ficoll-Hypaque as previously described[17].

2.2.2 Viability assays

Screening was performed in K562 and Jurkat cells ($5x10^4$cells/well) incubated with each pyrazoline for 24h. Cell viability was assessed by the MTT assay[18] (Sigma-Aldrich®) and optical density of the control groups (untreated cells) was considered as 100% of viable cells. The most cytotoxic compounds were selected and incubated with K562, Jurkat, B16-F10, Daudi and PBMC at different concentrations (1-100µM). The half-maximal inhibitory concentrations (IC_{50}) were calculated using GraphPad Prism 5 software and the selectivity index (SI) was calculated by dividing the IC_{50} obtained on PBMC by the IC_{50} obtained on K562, Jurkat, Daudi and B16-F10 cells. A high selectivity was considered as $SI \geq 5$, a moderate selectivity as $2 \leq IS < 5$ and a low selectivity as $IS < 2$[19]. Hemolysis test was performed on RBC using saline as a negative control (0% lysis), distilled water as a positive control (100% lysis) and increasing concentrations of selected pyrazolines. The hemolysis percentage (% hemolysis) was calculated as previously described [20].

2.2.3 Cell cycle analysis

K562 and Jurkat cells were incubated with the selected pyrazolines at their respective 24 h IC_{50} (**H3TM4**: 32.0 µM and 30.08 µM on K562 and Jurkat cells respectively; **H3TM6**: 55.7 µM and 33.7 µM on K562 and Jurkat cells respectively) for 12 h. Cell cycle analysis was assessed according to the kit protocol (PI/RNAse Solution Kit, Immunostep®). Analysis was performed by flow cytometry (FACSCantoII™, BD Immunocytometry Systems®) and data were analyzed using Infinicyt software version 1.7 (Cytognos®).

2.2.4 Apoptosis assays

Apoptotic cell death was first evaluated on K562 and Jurkat cells by ethidium bromide (EB) and acridine orange (AO) staining as previously described[17]. For the Annexin V assay, K562 and Jurkat cells were incubated for 12 h with the selected pyrazolines (24h IC_{50}) and then labeled with Annexin V–FITC (BD Biosciences®) before flow cytometry analysis. For the DNA fragmentation assay, DNA extraction of Jurkat cells was performed using a commercial kit (QIAGEN®) and DNA samples were separated by electrophoresis in 2% agarose gel embedded with EB (2µg/mL). All experiments were performed with pyrazolines **H3TM4** and **H3TM6** at the 24 h CI_{50} (**H3TM4**: 32.0 µM and 30.08 µM on K562 and Jurkat cells respectively; **H3TM6**: 55.7 µM and 33.7 µM on K562 and Jurkat cells respectively).

2.2.5 Mitochondrial membrane potential (MMP) and protein expression

K562 and Jurkat cells were incubated for 12 h with the selected pyrazolines at the 24 h CI_{50} (**H3TM4**: 32.0 µM and 30.08 µM on K562 and Jurkat cells respectively; **H3TM6**: 55.7 µM and 33.7 µM on K562 and Jurkat cells respectively).

For MMP evaluation, cells were incubated in a MitoView 633™ (Biotium®) solution (diluted 1:10.000) and for protein expression, cells were first fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences®). Subsequently, cells were stained with anti-Ki-67-FITC (BD Biosciences®), anti-Bax-PerCP (Santa Cruz Biotechnology®), anti-Bcl-2-FITC (Invitrogen®), anti-survivin-PE (Santa Cruz Biotechnology®), anti-AIF-FITC (Santa Cruz Biotechnology®), anti-FasR-PE (Santa Cruz Biotechnology®) and anti-caspase-3-PE (BD Biosciences®) for flow cytometry acquisition.

2.2.6 Ex vivo assays

Two PB and four BM samples obtained from patients with AL were collected before the first treatment at the diagnostic or at a relapse. MC were isolated as described above and incubated with increasing concentrations of the selected pyrazolines for 24 h before cell viability assessment.

2.3 Statistical analysis

Data are expressed as means ± SEM and each experiment has been repeated at least three times independently. Statistical analysis was conducted in GraphPad Prism 5 software using *t* test or ANOVA one-way and Bonferroni post-hoc test. $p \leq 0.05$ was considered as significant.

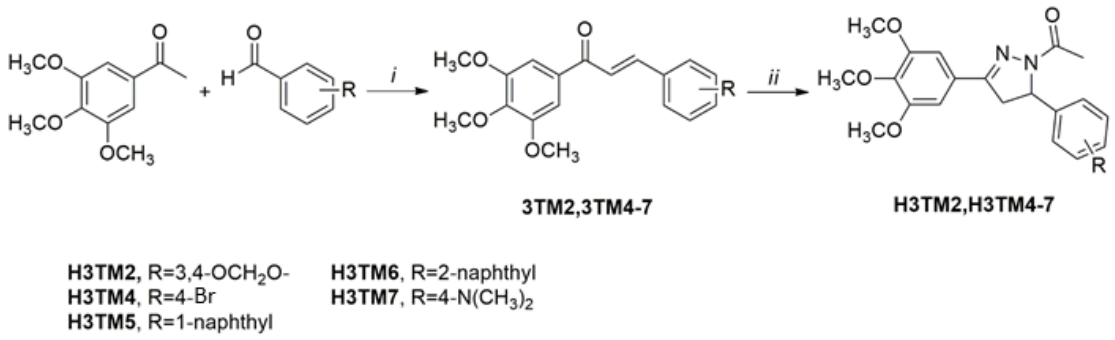
3 Results

3.1 Pyrazoline derivatives synthesis

Synthesis of the pyrazolines **H3TM2**, **H3TM4**, **H3TM5**, **H3TM6** and **H3TM7** was conducted in two steps (Fig.1A). The yields were considered good and found to be around 53 and 90%. Selected compounds **H3TM4** and **H3TM6** have not been previously reported in the literature and led to regular yields (41–85%). The ^{1}H NMR spectra revealed a typical ABX system, in which each hydrogen is shown as a double doublet with characteristic constants (*J*) and aromatic hydrogens at 6.5–7.5 ppm, according to the substituent in the aromatic group. In the ^{13}C NMR, methylene signals can be observed

between 20 and 45 ppm, a methoxy group between 55 and 60 ppm, and aromatic carbons and the pyrazoline C=N signal at 100–150 ppm. The signal observed between 168 and 189 ppm, attributed to the carbonyl group, confirms the acetylation performed during the synthesis.

Figure 1. Synthesis and screening of novel pyrazoline derivatives.



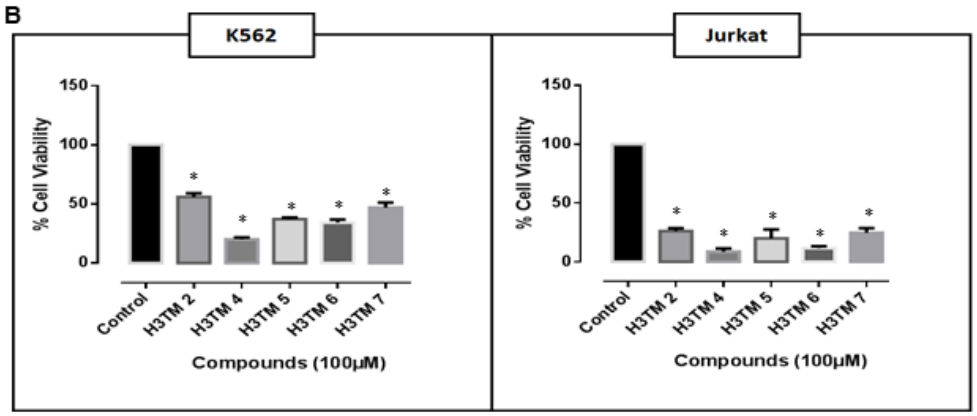


Figure 1. (A) Synthesis of precursor chalcones and pyrazolines (**H3TM2**, **H3TM4-7**). *(i)* ethanol, KOH 50% w/v, rt, 24h, HCl (10%); (ii) acetic acid, hydrazine hydrate, reflux 6h, NaHCO3(aq). (B) Screening of five pyrazolines (100µM) on AML K562 and ALL Jurkat cells after 24h incubation. * p ≤0.05 when compared to the control groups, one-way ANOVA followed by Bonferroni.

3.2 Pyrazoline derivatives have a good predicted oral bioavailability

All five pyrazolines synthesized have an MW lower than 500 g/mol, cLog P lower than five and PSA lower than 140 angstroms (Table 1). They also have fewer than 10 hydrogen acceptors, no hydrogen donors and flexible bonds, which is in agreement with the physicochemical parameters considered ideal for a predicted good oral bioavailability, as they have adequate solubility and permeability [15, 16].

Table 1. Physicochemical properties. Theoretical prediction of pyrazolines **H3TM2**, **H3TM4-7** properties according to Lipinski et al. (2001) and Veber et al. (2002).

Properties/ Compounds	MW	cLogP	H -- acceptors	H – donors	Rotatable bonds	PSA	Violations
H3TM2	398.2	2.4	8	0	5	78.8	0
H3TM4	433.3	3.4	6	0	5	60.4	0
H3TM5	404.5	3.7	6	0	5	30.4	0
H3TM6	404.5	3.7	6	0	5	60.4	0
H3TM7	397.5	2.7	7	0	6	63.6	0
***Lipinski et al. (2001)**	≤ 500	≤ 5	≤ 10	≤ 5	-	-	-
***Veber et al. (2002)**	-	-	≤ 12	≤ 12	≤ 10	≤ 140	-

3.3 Pyrazoline derivatives are cytotoxic against neoplastic cells

All five synthetic pyrazolines were cytotoxic to K562 and Jurkat cells at 100µM (Figure 1B). The most cytotoxic and unpublished compounds, **H3TM4** and **H3TM6** reduced the cell viability of K562, Jurkat, Daudi and B16-F10 in a concentration- and time-dependent manner (Supp.Fig.1); the IC_{50} values are shown in Table 2.

Table 2. IC_{50} values. IC_{50} for **H3TM4** and **H3TM6** on K562, Jurkat, Daudi, B16-F10 and non neoplastic cells (PBMC).

H3TM4 IC_{50} (µM)					
Cells/Time	K562	Jurkat	Daudi	B16-F10	PBMC
24h	32.0 ± 1.1	30.8 ± 0.6	49.8 ± 1.4	98.7 ± 1.6	≈ 339.8 ± 16.6
48h	16.2 ± 0.3	17.2 ± 0.6	34.8 ± 1.2	72.7 ± 2.4	≈ 287.1 ± 6.5
72h	15.1 ± 1.0	15.6 ± 0.5	33.0 ± 0.8	62.5 ± 1.3	≈ 117.5 ± 2.4
H3TM6 IC_{50} (µM)					
Cells/Time	K562	Jurkat	Daudi	B16-F10	PBMC
24h	55.7 ± 0.8	33.7 ± 1.2	46.2 ± 1.5	72.3 ± 2.1	≈ 496.7 ± 23.2
48h	21.9 ± 0.4	27.7 ± 0.7	20.2 ± 0.8	56.6 ± 1.2	≈ 104.7 ± 2.9
72h	15.5 ± 0.5	25.3 ± 0.6	5.1 ± 0.9	23.3 ± 0.7	55.3 ± 1.6

3.4 Selected pyrazolines H3TM4 and H3TM6 are highly selective for tumor cells

Table 2 shows that the selected pyrazolines did not reduce the viability of PBMC. In fact, when compared to non-neoplastic cells, **H3TM4** and **H3TM6** were highly selective for tumor cell lines, as demonstrated by the SI displayed in Table 3. Additionally, significant hemolysis was observed only at concentrations 12 and 15 times higher than those found in AL cells (Table 4).

Table 3. SI for H3TM4 and H3TM6. SI was calculated by the ratio $IC_{50\ PBMC}$/ $IC_{50\ Neoplastic\ cells\ ,}$ obtained after 24h incubation.

	H3TM4	**H3TM6**
Jurkat	≈ 10.6	≈ 14.7
K562	≈ 11.0	≈ 8.9
Daudi	≈ 6.8	≈ 10.7
B16-F10	≈ 3.4	≈ 6.9

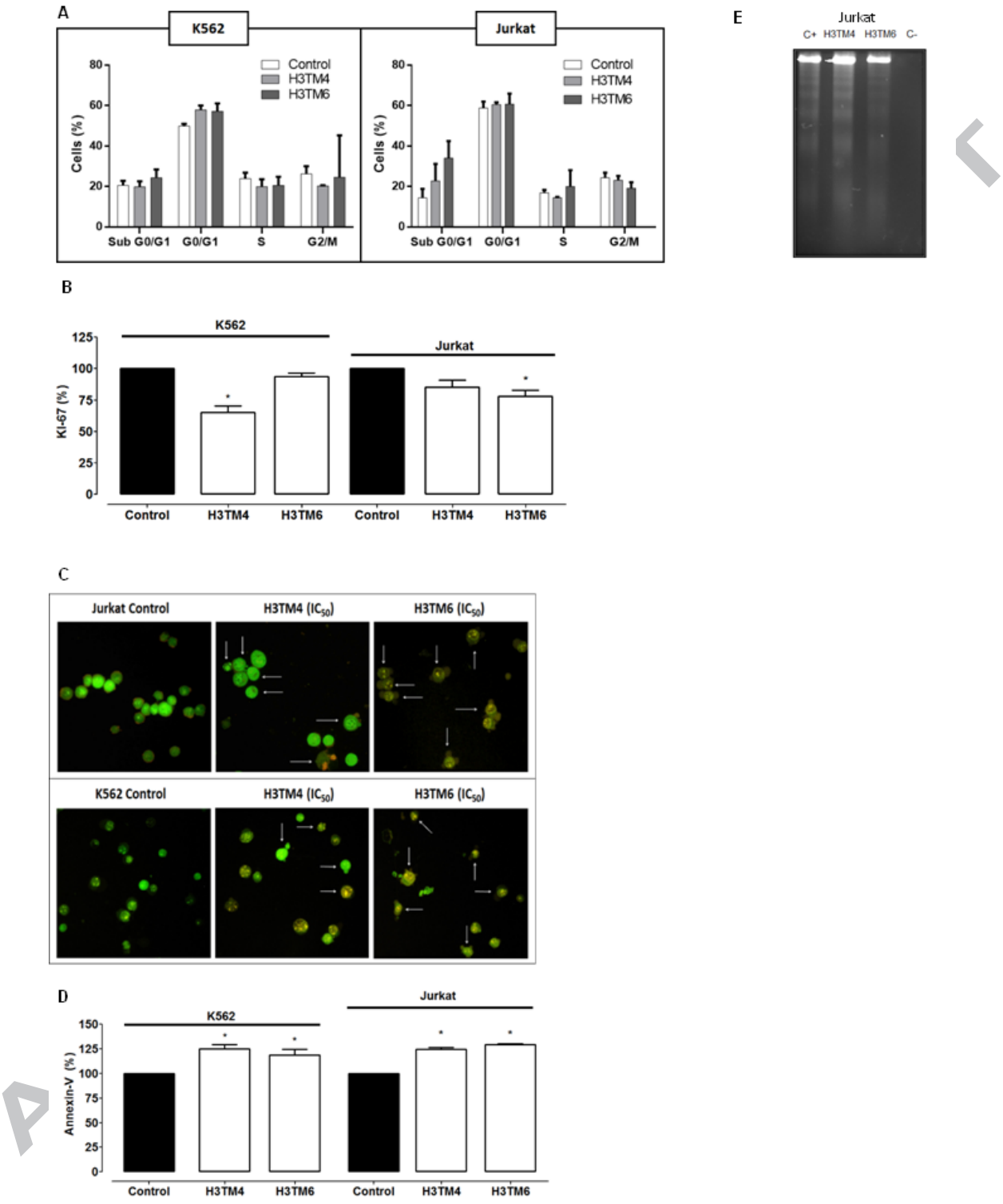
Table 4. Hemolysis on RBC. Hemolysis percentage induced by **H3TM4** and **H3TM6** when compared with the negative control (0% hemolysis).

% HEMOLYSIS		
Concentration	**H3TM4**	**H3TM6**
1X IC_{50}	0.9 ± 0.6	1.9 ± 1.2
2X IC_{50}	1.3 ± 1.0	1.5 ± 1.2
3X IC_{50}	1.6 ± 0.5	1.6 ± 1.3
9X IC_{50}	6.0 ± 5.7	2.4 ± 1.8
12X IC_{50}	12.9 ± 3.5*	3.2 ± 0.4
15X IC_{50}	15.8 ± 5.1*	6.4 ± 1.5*

3.5 H3TM4 and H3TM6 induce cell cycle arrest, apoptosis and reduce cell proliferation

A significantly increased percentage of K562 cells at the G0/G1 phase was observed after treatment with **H3TM4** and **H3TM6** (8.0% and 7.4%, respectively) (Fig.2A). In Jurkat cells, a mild but non-significant increase was seen at the S phase. Treatment with **H3TM4** and **H3TM6** also reduced expression of the cell proliferation marker KI-67 in both cell lines (Fig.2B). Morphological changes resulting from the cell death induced by **H3TM4** and **H3TM6** revealed apoptotic characteristics, such as membrane blebbing, chromatin condensation and membrane integrity loss (Fig.2C). The percentage of Annexin-V positive K562 cells increased by 25.0% and 18.8% after treatment with **H3TM4** and **H3TM6**, respectively, while an increase of 24.6% and 29.2% was observed in Jurkat cells (Fig.2D). Finally, apoptosis, confirmed as the typical "ladder pattern", was detected in treated Jurkat cells (Fig.2E).

Figure 2. Cell cycle and apoptosis assays.

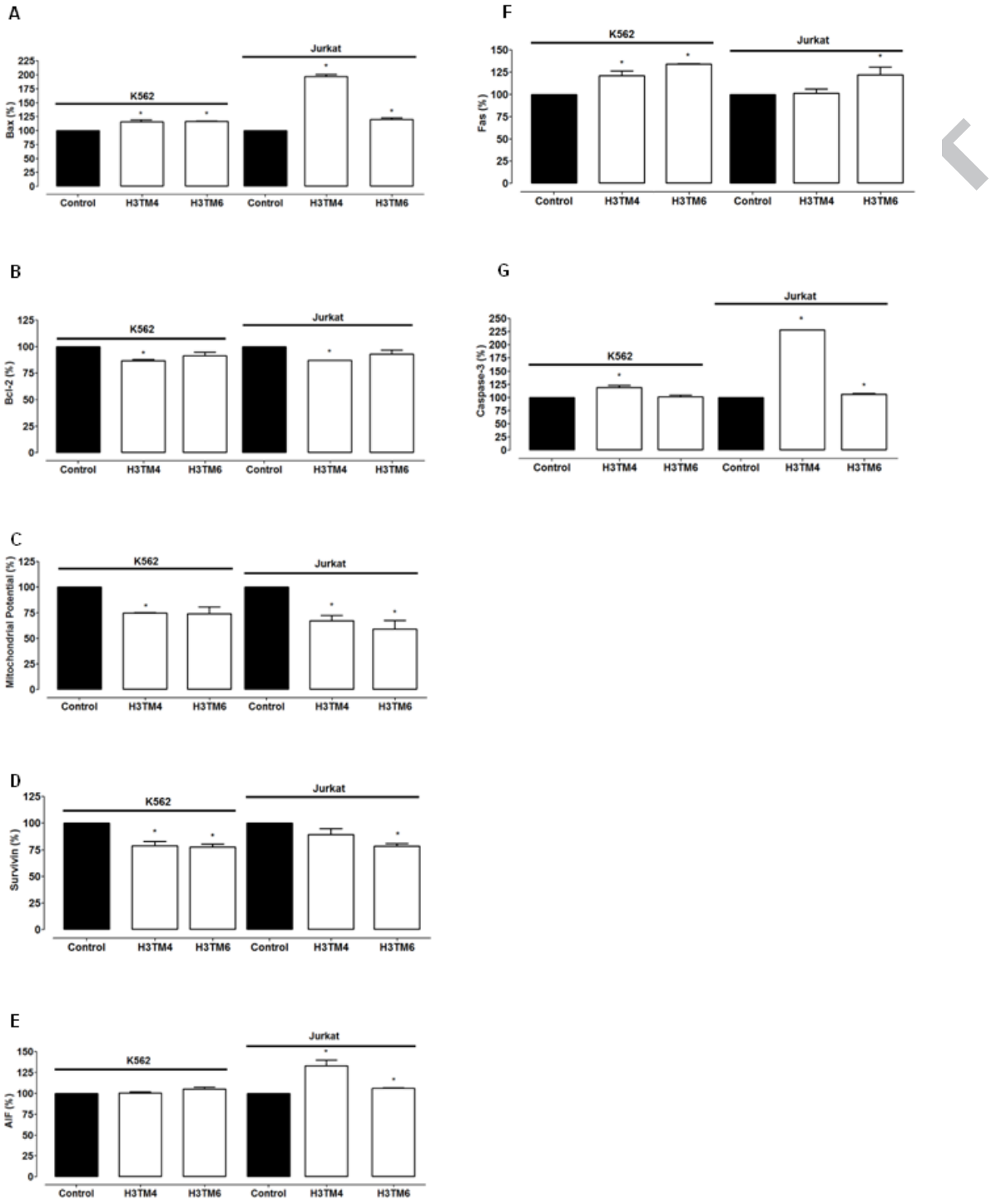


(A) Cell cycle analysis of K562 and Jurkat cells treated with **H3TM4** and **H3TM6** (24 h IC_{50}), stained with PI and evaluated by flow cytometry. (B) Ki67 expression (%) was determined by flow cytometry after labeling with anti-Ki67. (C) Apoptotic K562 and Jurkat cells are indicated by the white arrows after AO/EB staining. (D) Annexin-V positive K562 and Jurkat cells (%) were quantified by flow cytometry. (E) DNA fragmentation assay on Jurkat cells. * $p \leq 0.05$ when compared to control groups, *t*-test or ANOVA one-way followed by Bonferroni.

3.6 H3TM4 and H3TM6 induce intrinsic and extrinsic apoptosis on AL cells

Pyrazolines **H3TM4** and **H3TM6** significantly increased Bax expression in K562 (15.8% and 16.8%) and Jurkat cells (101.4% and 20.5%) (Fig.3A), while the anti-apoptotic protein Bcl-2 was significantly reduced only after treatment with **H3TM4** (K562: 13.4%, Jurkat: 12.9%) (Fig.3B). The percentage of K562 cells with an intact MMP was reduced by 25.3% and 26.0% after treatment with **H3TM4** and **H3TM6**, respectively (Figure 3C), while Jurkat cells with an intact MMP reduced by 32.9% and 41.0%. The selected pyrazolines also decreased survivin expression in both K562 (**H3TM4:** 21.2%, **H3TM6:** 22.2%) and Jurkat cells (**H3TM4**: 10.7%; **H3TM6**: 21.4%) (Figure 3D), and induced AIF release in ALL cells (**H3TM4**: 33.2%; **H3TM6**: 6.0%) (Figure 3E). K562 cells treated with **H3TM4** and **H3TM6** showed increased FasR expression (21.3% and 34.3%, respectively), while only treatment with **H3TM6** modulated FasR in Jurkat cells (30.3%) (Figure 3F). Finally, treatment with **H3TM4**, but not **H3TM6**, significantly increased active caspase-3 expression (20.0%) in K562 cells, whereas both compounds modulate this protein in Jurkat cells (**H3TM4**: 128.3% and **H3TM6**: 6.6%) (Figure 3G).

Figure 3. Effect of selected pyrazolines in the apoptotic machinery.



(A-B) K562 and Jurkat cells incubated with **H3TM4** and **H3TM6** were labeled with anti-Bax-PerCP (A) and anti-Bcl-2-FITC (B). (C) MMP % of K562 and Jurkat cells by MitoView. (D-G) K562 and Jurkat labeled with anti-Survivin-PE (D), anti-AIF-FITC (E), anti-FasR-PE (F) and anti-caspase-3-PE (G). All experiments were acquired by flow cytometry. * $p \leq 0.05$ when compared to the control groups using *t*-test.

3.7 Pyrazolines H3TM4 and H3TM6 are cytotoxic to AL patients' cells

Pyrazolines **H3TM4** and **H3TM6** reduced the cell viability of all PB and BM samples collected from AL patients in a concentration-dependent manner (Supp.Fig.2). Different IC_{50} values were found for each case, as demonstrated in Table 5.

Table 5. Patients information and results. Clinical information about the seven AL patients and IC_{50} values calculated after MC incubation with **H3TM4** and **H3TM6**.

Patient	Age (years)	Sample	Diagnosis	Leukocytes/ mm³	Blast cells (%)	H3TM4 IC_{50} (µmol/l)	H3TM6 IC_{50} (µmol/l)
1	52	PB	AML inv(16)	7090	45	11.8 ± 1.3	37.1 ± 1.1
2	28	PB	B-ALL	9590	50	141.6 ± 2.7	139.0 ± 3.6
3	81	PB	Early T-cell precursor ALL	-	76	71.3 ± 1.8	46.0 ± 1.0
4	56	PB	AML without maturation	-	83	40.2 ± 1.9	27.7 ± 0.7
5	67	BM	AML secondary to MDS	5710	38	31.0 ± 0.9	16.2 ± 0.6
6	33	BM	AML t(8;21)	8550	27	98.2 ± 2.8	44.6 ± 1.2
7	31	BM	PML t(15;17) Bcr3+ FLT3 DIT+	115070	96	39.9 ± 0,6	29.0 ± 0.8

PML – Acute promielocytic leukemia
MDS – Myelodysplastic syndrome

4 Discussion

In this report, we have provided new information about the effects of a series of specific and cytotoxic pyrazoline derivatives on malignant cells. The five compounds fit the oral bioavailability parameters, which are important for the rational investigation of new drugs [21] as this may contribute to future structure-activity relationship (QSAR) studies. Considering the chemical structures of these pyrazolines, the 3,4,5-trimethoxy substitution on the A ring seems to be responsible for their cytotoxic activity. As already stated in the literature, this group is essential for the antitumor activity of chemotherapeutics like vincristine, used against ALL [3]. In addition, the presence or absence of methoxyl groups (OCH_3) linked to the A ring characterizes the lipophilicity variation of the molecule and its presence is related to different biological activities [22, 23]. The presence of electron density donor groups (OCH_3) on the A ring also seems to be relevant. At the B-ring, the presence of an electronegative (electron acceptor) group as the bromine atom (**H3TM4**), may be responsible for the increased cytotoxic activity of

this compound, to the both cell lines. Furthermore, hydrophobic substituents as B-ring (**H3TM5** and **H3TM6**) also seems to be relevant for the cytotoxicity of the compounds, especially the 2-naphthyl group (**H3TM6**) against Jurkat cells; the naphthyl group can interact with other hydrophobic rings by interactions between the π orbitals, which may favor their cytotoxic activity [24, 25]. On the other hand, an electron-donor group as a dimethylamine (**H3TM7**) or a heteroatom ring as 3,4-methylenedioxy (**H3TM2**) in the B ring caused reduction in cytotoxic activity, especially for K562 cells.

Pyrazolines **H3TM4** and **H3TM6**, the two more cytotoxic compounds, were active against T-ALL Jurkat, AML K562 and BL Daudi cells; the different IC_{50} values might be explained by the heterogeneity of hematological malignancies [19]. The K562 cell line originates from an AL with Bcr/abl oncogene, which is largely responsible for its resistance to chemotherapy [26, 27], while BL affects mature B lymphoid cells and is also a very aggressive disease [10, 28]. Considering that therapeutic protocols involve several chemotherapy cycles, these results are very promising, especially when taking into account that some cells are usually resistant to conventional chemotherapeutics in the first 24 h [26]. Similar studies evaluated the effect of B-ring substituted steroidal pyrazoline derivatives and glycoside derivatives of ferrocenyl-pyrazolines on AL cells and found IC_{50} values ranging from 10.6 μM to >200 μM [27, 29]. Interestingly, **H3TM4** and **H3TM6** were not effective against non-tumor cells, which suggests that these pyrazolines are highly selective for hematological malignancies and have a high blood biocompatibility. Since the non-selective toxicity of currently available chemotherapeutic agents is an important factor influencing treatment failure [6, 11, 17], this was considered a very promising result.

To date, few studies elucidating the anti-leukemic effect of pyrazolines have been described in the literature. Bao et al. [30] reported that a pyrazoline derivative induced G0/G1 arrest, while Santos-Bubniak et al. [20] reported cell cycle arrest at the S phase. Cell cycle arrest at the S phase, as seen in Jurkat cells treated with **H3TM4** and **H3TM6**, suggests interference with DNA replication [31], whereas G0/G1 arrest, as seen in K562 cells, could be related to p53 activation. p53 acts in the first checkpoint, and is fundamental to genome integrity, as it allows DNA repair mechanisms and the removal of damaged cells through apoptosis [32, 33]. Similarly, as uncontrolled cell proliferation is highlighted as one of the main features of oncogenesis, Ki67 expression is often used *in vitro* and in clinics as a proliferation marker [34, 35]. **H3TM4** and **H3TM6** decreased KI67 expression in AL cells, suggesting that they might modulate neoplastic cell proliferation.

Cell death may occur through several regulated and non-regulated mechanisms [36]. We have demonstrated that pyrazolines **H3TM4** and **H3TM6** induce apoptosis in AML and ALL cells, which is in agreement with other studies found in the literature [20, 30].

The Bcl-2/Bax ratio after **H3TM4** and **H3TM6** treatment demonstrates Bax predominance in relation to Bcl-2 in both K562 and Jurkat cells. According to the literature, Bax and Bcl-2 modulation induces pores opening at the mitochondrial membrane, resulting in MMP loss and the release of pro-apoptotic factors to the mitochondria and the cytosol [37, 38]. Treatment with **H3TM4** and **H3TM6** increased AIF expression in Jurkat but not K562 cells, suggesting that, in AML cells, other proteins might be involved in cell death, such as cytochrome *c*, SMAC and EndoG. These results are in agreement with two other studies that reported the activation of intrinsic apoptosis by pyrazolines, as evidenced by an increase in Bax and AIF expression, decrease in Bcl-2 expression [20] and MMP loss [20, 30]. Extrinsic apoptosis also seems to be involved in cell death induced by **H3TM4** and **H3TM6** on AL cells, as evidenced by increased FasR expression. FasR is one of the membrane receptors that triggers intracellular signaling cascades, culminating in the activation of caspase-8 and effector caspases-3 and -7, which results in apoptosis independent of the mitochondrial signaling pathway [39, 40]. Intrinsic and extrinsic apoptosis led to caspase-3 activation in Jurkat cells, whereas only **H3TM4** was able to increase active caspase-3 expression in K562 cells. The non-modulation of caspase-3 by **H3TM6** on K562 cells suggests that this pyrazoline might induce a caspase-independent cell death with apoptotic characteristics; however, further studies are needed to elucidate this question. Additionally, **H3TM4** and **H3TM6** decreased survivin expression in K562 cells and **H3TM6** did the same in Jurkat cells. Studies suggest that survivin is related to radiation and chemotherapy resistance in clinics and this protein inhibition may lead to a greater sensitivity of neoplastic cells to current cancer treatments [41, 42].

Finally, **H3TM4** and **H3TM6** were evaluated in PB and MC cells collected from patients with different AL subtypes. Different IC_{50} values have been found, which was expected given that leukemias are heterogeneous diseases that react differently to treatment [10, 26, 27]. It is important to highlight that of all patients included in this study only Patient **7** had relapsed when the cells were obtained. This patient was diagnosed with PML with a t(15;17) Bcr3 positive and FLT3/DIT mutation. The presence of a DIT/FLT3 mutation in patients with PML t(15;17) is associated with a very poor prognosis [10]. The results obtained for **H3TM4** and **H3TM6** showed that they were both effective against these

resistant cells. Since not every PML patient responds well to conventional treatment, it is very important to find new cytotoxic molecules against these AL subtypes. Pyrazolines **H3TM4** and **H3TM6** were also cytotoxic against other aggressive AL subtypes such as AML secondary to myelodysplastic syndrome (MDS), early T-cell precursor ALL and AML without maturation [10]. It is evident that the small sample number does not allow us to draw any definitive conclusion about the effect of pyrazolines on each AL subtype. However, the results obtained so far are quite promising, as they show that **H3TM4** and **H3TM6** are cytotoxic not only against neoplastic cell lines but also against cells collected from patients with different AL subtypes. So far, no studies have been found reporting the evaluation of pyrazoline in AL patient cells.

5 Conclusion

This study demonstrates that the previously unpublished pyrazolines **H3TM4** and **H3TM6** are selective and cytotoxic to different hematological malignancies and that the death mechanisms involve cell cycle arrest, the inhibition of cell proliferation and the induction of intrinsic and extrinsic apoptosis. Altogether, the results suggest that these novel compounds are promising candidates for the development of efficient new prototypes against AL.

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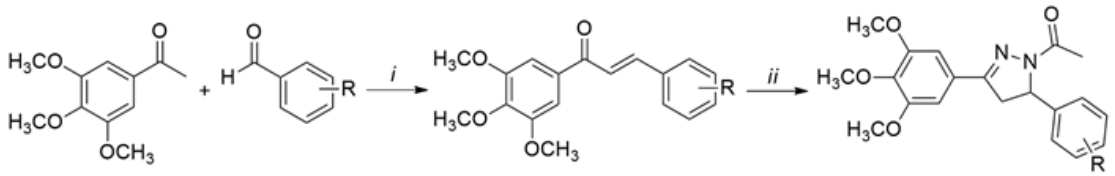
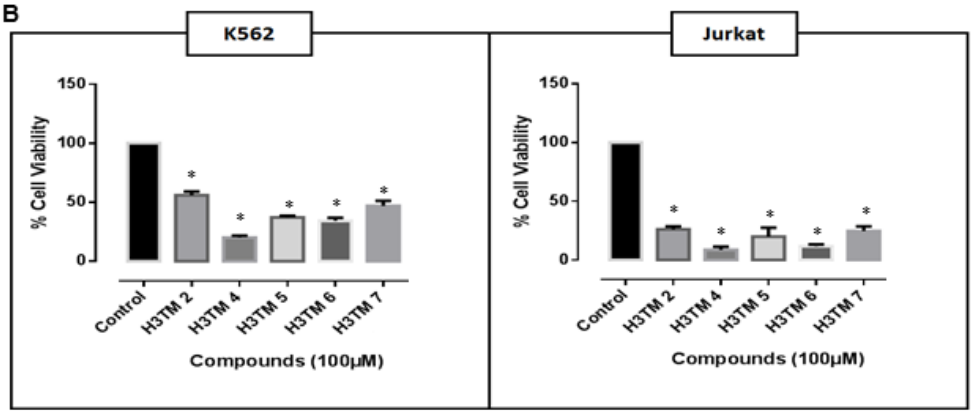
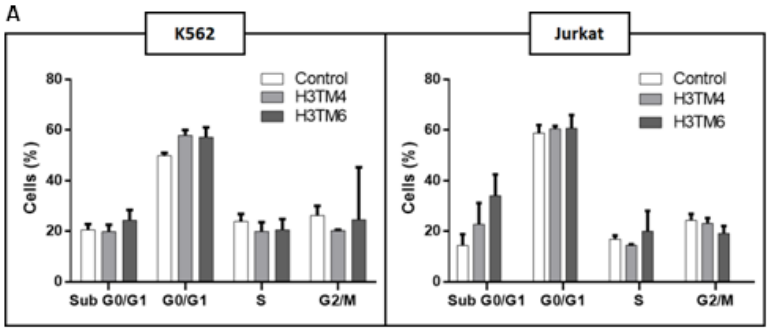


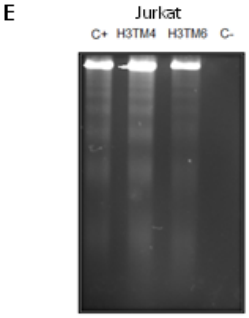
3TM2,3TM4-7 H3TM2,H3TM4-7

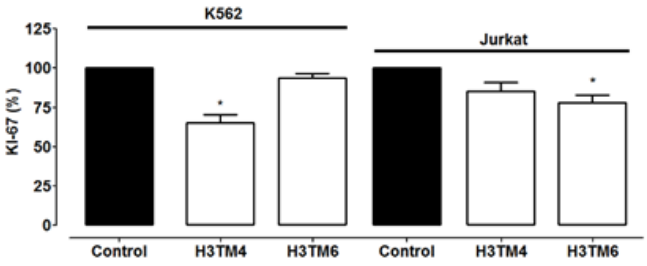
H3TM2, R=3,4-OCH_2O- **H3TM6**, R=2-naphthyl
H3TM4, R=4-Br **H3TM7**, R=4-$N(CH_3)_2$
H3TM5, R=1-naphthyl

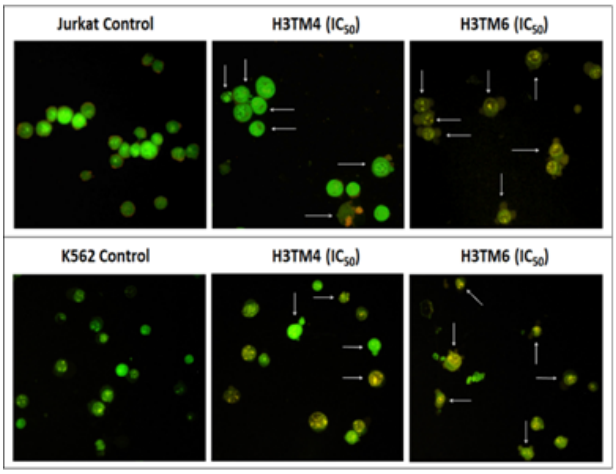
B

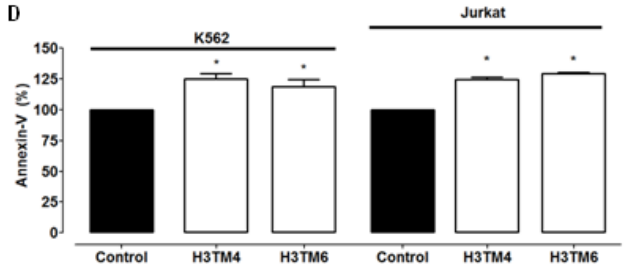


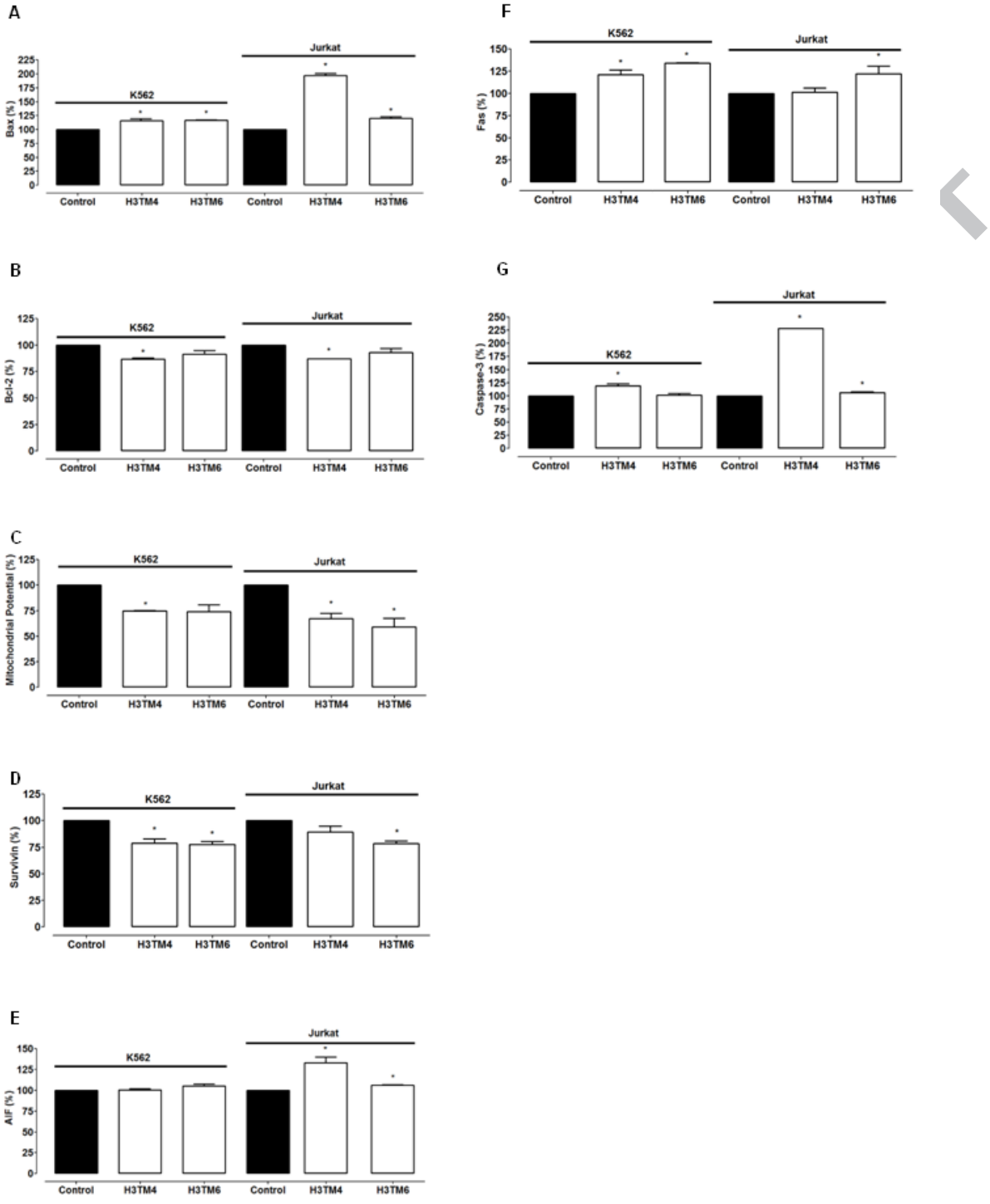


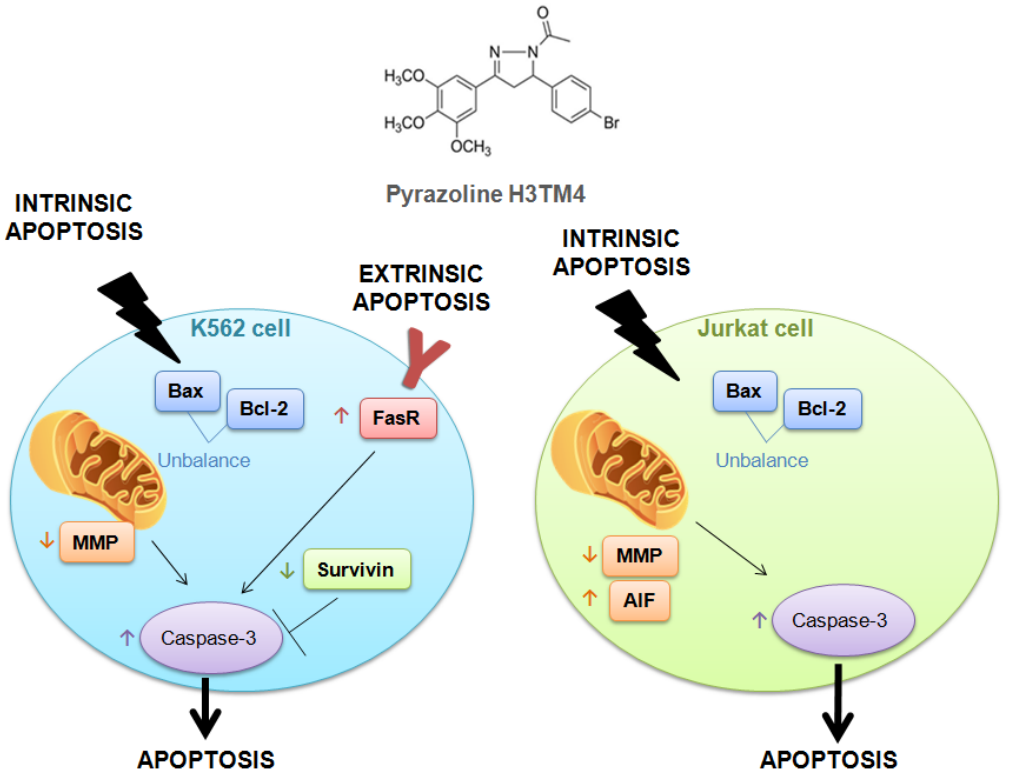


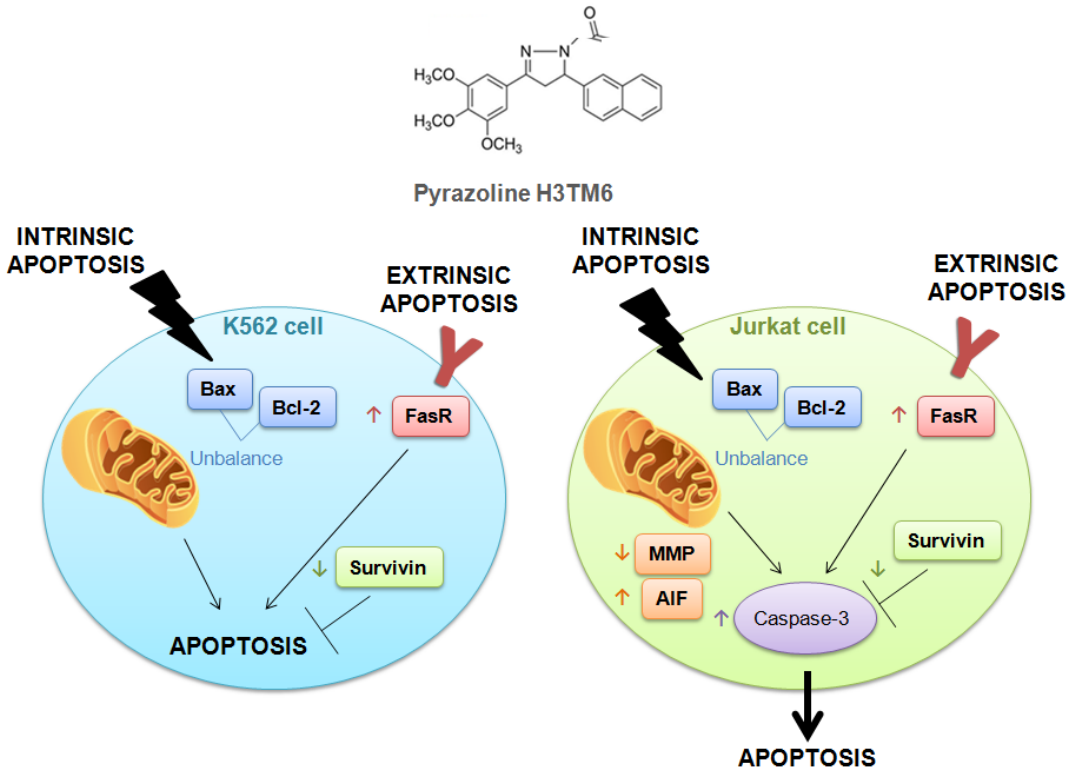












HIGHLIGHTS

- **H3TM4** and **H3TM6** are highly cytotoxic and selective to several neoplastic cell lines.
- **H3TM4** and **H3TM6** have physicochemical properties for a good oral bioavailability.
- **H3TM4** and **H3TM6** induce cell cycle arrest and decrease cell proliferation.
- Cell death by **H3TM4** and **H3TM6** involves intrinsic and extrinsic apoptosis.
- **H3TM4** and **H3TM6** kill cancer cells from patients with different acute leukemia subtypes.