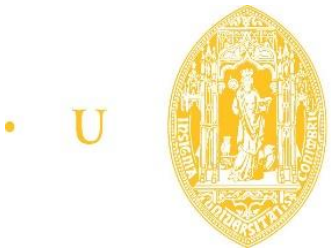

FMUC FACULDADE DE MEDICINA
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THE EFFECT OF GAMBOGIC ACID AND SILIBININ IN ACUTE MYELOBLASTIC LEUKEMIA

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The effect of Gambogic Acid and Silibinin in Acute Myeloblastic Leukemia

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Abstract

Acute myeloblastic leukemia (AML) is a malignant hematological disease characterized by an uncontrolled proliferation with subsequent accumulation of immature myeloid cells in the bone marrow. Despite the development of new therapies for treating AML, the standard treatment relies in intensive chemotherapy with combination of several drugs. Natural compounds such as gambogic acid (GA), extracted from *Garcinia hanburyi*, and silibinin (SLB), from *Silybum marianum*, showed an antitumoral effect in many neoplasms, by induction of apoptosis and differentiation as well as proliferation inhibition. However, the effect of these two compounds on AML is still unclear.

Therefore, this study aimed to evaluate the therapeutic potential of GA and SLB in *in vitro* models of AML.

With this purpose, we used two *in vitro* AML models, KG-1 and THP-1 cells, incubated in absence and presence of GA and SLB, in monotherapy (single and daily administration) and in combination therapy. Metabolic activity was evaluated by resazurin assay. Cell death was assessed by optic microscopy (May-Grünwald-Giemsa staining) and flow cytometry (FC), using the Annexin V and 7-AAD double staining. Cell cycle and caspase-3 activation were evaluated by FC. The statistical analysis was performed, considering a significance level of 95%.

Results show that GA and SLB decreased the metabolic activity in a time, dose and cell line dependent manner. After 72h of incubation, IC_{50} of GA was 338 nM in KG-1 cells and 453 nM in THP-1, while IC_{50} for SLB was 33 µM and 75 µM in KG-1 and THP-1, respectively. Daily administration was more effective in KG-1 cells, whereas combination therapy presented a synergistic effect in both cell lines. The drugs induced cell death by apoptosis, proved by increased caspase-3 activation and morphological analysis. Cell cycle analysis demonstrated that SLB caused cell cycle arrest in G_0/G_1, while GA had no cytostatic effect.

In conclusion, our results suggest that GA and SLB may constitute new potential therapeutic approaches in acute myeloblastic leukemia, depending on cellular characteristics and/or drug administration scheme.

Keywords: Acute Myeloblastic Leukemia; Gambogic Acid; Silibinin; Apoptosis; Caspase-3

Resumo

A leucemia mieloblástica aguda (LMA) é uma neoplasia hematológica caracterizada pela proliferação descontrolada de progenitores hematopoiéticos com acumulação na medula óssea. Apesar do desenvolvimento de novas terapêuticas para o tratamento da LMA, este ainda se centra na quimioterapia intensiva com combinação de vários fármacos. Neste sentido, torna-se necessário introduzir novas estratégias terapêuticas. Compostos naturais como o ácido gambógico (GA), extraído da planta *Garcinia hanburyi*, e a silibinina (SLB), da planta *Silybum marianum*, têm demonstrado efeito antitumoral em diferentes neoplasias, sendo o seu efeito mediado por indução da apoptose, da diferenciação celular e pela inibição da proliferação. Contudo, o efeito destes dois compostos na LMA ainda não está esclarecido.

Este trabalho avaliou o potencial terapêutico do GA e da SLB, em monoterapia e em combinação terapêutica, em modelos *in vitro* de LMA.

Deste modo, as linhas celulares KG-1 e THP-1 foram incubadas na ausência e presença de GA e SLB, em monoterapia (administração única e fracionada) e em combinação terapêutica. A atividade metabólica foi avaliada pelo teste da resazurina. A morte celular foi avaliada por microscopia ótica (coloração May-Grünwald-Giemsa) e citometria de fluxo (CF), através da dupla marcação com Anexina V e 7-AAD. O ciclo celular e a ativação da caspase-3 foram também avaliados por CF. Foi efetuada a análise estatística adequada considerando-se um nível de significância de 95%.

Os resultados mostram que o GA e a SLB reduzem a atividade metabólica de forma dependente do tempo de incubação, da dose e da linha celular. Após 48 horas de incubação, o IC_{50} do GA foi de 338 nM nas KG-1 e 453 nM nas THP-1, enquanto o IC_{50} da SLB foi de 33 μM e 75 μM nas KG-1 e nas THP-1, respetivamente. A administração fracionada mostrou-se mais eficaz nas células KG-1, enquanto que a terapia combinada apresentou um efeito sinérgico em ambas as linhas celulares. Os compostos em estudo induziram morte celular por apoptose, comprovada por aumento da ativação da caspase-3 e pela análise morfológica. A análise do ciclo celular revelou que a SLB provoca bloqueio do ciclo em G_0/G_1. Todavia, o GA não apresentou efeito citostático.

Concluindo, os nossos resultados sugerem que o ácido gambógico e a silibinina poderão representar potenciais abordagens terapêuticas na leucemia mieloblástica aguda, dependendo de características celulares e/ou do esquema de administração.

Palavras-chave: Leucemia Mieloblástica Aguda; Ácido Gambógico; Silibinina; Apoptose; Caspase-3

Abbreviations

1,25D: 1, 25-dihydroxyvitamin D3
7-AAD: 7-amino-actinomycin D
ALL: Acute lymphoblastic leukemia
AML: Acute myeloblastic leukemia
ATC: Anaplastic thyroid cancer
ATCC: American Type Culture Collection
AV: Annexin-V
CAR T: chimeric antigen receptor T cells
CI: Combination Index
CML: Chronic myeloid leukemia
DA: Daily administration
FBS: Fetal bovine serum
FC: Flow cytometry
FDA: U.S. Food and Drug Administration
GA: Gambogic acid
GO: Gemtuzumab ozogamicin
HCC: Hepatocellular carcinoma
HiDAC: High-dose cytarabine
HSCT: Hematopoietic stem cell transplantation
LMA: Leucemia mieloblástica aguda
PARP-1: poly (ADO-ribose) polymerase 1
PI: Propidium iodide
PML: Promyelocytic leukemia
ROS: Reactive oxygen species
RPMI 1640: Roswell Park Memorial Institute 1640 medium
SLB: Silibinin
STAT-3: Signal transducer and activator of transcription-3
WHO: World Health Organization

Introduction

Acute myeloblastic leukemia (AML) is a malignant hematological disease characterized by an uncontrolled proliferation of multipotent hematopoietic progenitors with subsequent accumulation of immature myeloid cells in the bone marrow. It is usually caused by genetic or epigenetic alterations that arrest differentiation of hematopoietic stem cells.[1] WHO classifies AML according to morphological criteria, cytogenetic, molecular genetics, immunophenotype and clinical information, categorizing clinically significant disease entities.[2] Currently, AML is the most common acute leukemia in adults and its diagnose relies on the presence of 20% or more myeloid blasts in the bone marrow and/or peripheral blood.[2,3] The exceptions to this criteria are related to cytogenetic alterations, such as t(8;21)(q22;q22), inv(16)(p12q22) or t(16;16)(p13;q22) and t(15;17)(q22;q12).[3]

Presently, standard treatment of AML is based on intensive chemotherapy divided in an induction, consolidation and, rarely, maintenance.[2] Induction chemotherapy follows a "7+3" regimen, consisting of a continuous intravenous infusion of cytarabine for 7 days (100 or 200 mg/m^2 daily) and 3 daily doses of an anthracycline, typically daunorubicin (45–90 mg/m^2) or idarubicin (12 mg/m^2 daily).[3,4] The induction regimen is followed by the consolidation therapy, aiming to clear remaining leukemia cells, once patients have reached remission, both clinical and hematological.[2] It usually comprises a high dose of cytarabine (HiDAC), twice daily at a 3 g/m^2 dose on days 1, 3, and 5, for patients of young age and who are not undergoing a stem cell transplantation.[4] The allogeneic hematopoietic stem cell transplantation (HSCT) offers the best possibility of avoiding recurrence of AML, but has significant morbidity and mortality associated, particularly in older patients.[2,4] HSCT is therefore indicated for patients with intermediate and poor prognosis AML with an HLA-identical sibling or HLA-matched unrelated donor, provided their age and performance status.[2,4] Other therapies, targeted to specific AML subgroups, were established.[4]

However, despite the options available and the usual favorable initial response to chemotherapy, the prognosis for most patients remains poor, especially due to primary resistance and frequent relapse, with the majority of patients relapsing and dying of their disease within 2 years of remission.[2,5,6] In this context, other therapies have been approved, namely in targeted cancer therapies as *FLT-3*[7] and *IDH* inhibitors and in immunotherapy with monoclonal antibodies,[8] targeting mainly AML patients with specific mutations and/or relapsed or refractory AML. In promyelocytic leukemia (PML), retinoic acid and arsenic trioxide were approved.[5] However, it is still necessary to investigate new therapeutic

approaches to overcome the issue of resistance and relapse in AML patients. With that purpose, it has been studied the effect of many natural compounds.

Gambogic acid (GA) is a molecule extracted from the plant *Garcinia hanburyi*, commonly used in traditional Chinese medicine. It has demonstrated its antitumoral effect in multiple cancer cell lines, namely in AML,[9,10] chronic myeloid leukemia (CML),[11] anaplastic thyroid cancer (ATC),[12] bladder,[13] colorectal,[14] and pancreatic[15] cancer. Unlike other chemotherapies, GA has shown less toxicity to normal tissues. Its effect is achieved throw several pathways, including apoptosis induction, antiangiogenesis, and inhibition of topoisomerase-II.[11-15]

Silibinin (SLB) is obtained from the plant *Silybum marianum* and Its antitumoral effect has been demonstrated in cancer cell lines, such as AML,[1,5,16] glioblastoma,[17] gastric cancer,[18] hepatocellular carcinoma[19] and prostate cancer[20]. Regarding prostate cancer, it has been approved by the U.S. Food and Drug Administration for phase II clinical trials.[21] The mechanism of SLB in tumoral cells is based in the induction of apoptosis and differentiation, proliferation inhibition and targeting angiogenesis and cancer cell metabolism.[17-19]

Nevertheless, previous studies in AML using GA or SLB only tested single administration schemes or combinations with other drugs, and not daily administration nor combination with both drugs. Therefore, the effects of these schemes using GA and SLB in AML remains unclear. Thus, the aim of this work was to assess the therapeutic effect of different administration schemes of gambogic acid and silibinin in AML, namely in single, daily fractioned administration and in combination therapy.

Material and Methods

Cell Culture

In this study we used two acute myeloblastic leukemia (AML) cell lines, KG-1 and THP-1, which were purchased from American Type Culture Collection (ATCC). KG-1 cells were obtained from the bone marrow of a 59 years-old male patient with erythroleukemia. THP-1 cells were obtained from the peripheral blood of a 1-year-old infant with acute monocytic leukemia. The cells were maintained in Roswell Park Memorial Institute 1640 (RPMI 1640) medium and supplemented with 10% fetal bovine serum (FBS), 2 mM of L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Gibco, Invitrogen), at 37ºC in a humidified atmosphere with 5% CO_2.

Cellular metabolic activity

To evaluate the effect of GA and SLB on cellular metabolic activity, the two cell lines were cultured in the presence and absence of increased concentrations of these compounds in monotherapy and in combination therapy during 72 hours. The monotherapy treatment included two therapeutic schemes: single and daily fractioned administration.

Gambogic acid (GA) and silibinin (SLB) were purchased from APExBIO and dissolved in dimethyl sulfoxide (DMSO). Both cell lines were incubated with GA in concentrations ranged from 5 nM to 1000 nM and SLB from 0.5 µM to 250 µM. In addition to single monotherapy, cells were also incubated for 72 hours with a daily fractioned administration of GA in a concentration of 50 nM and SLB in a concentration of 15 µM for KG-1 and 25 µM for THP-1. A combination therapy of GA and SLB, with the concentrations of 50 nM and 15 µM, respectively, was also tested.

Metabolic activity was analyzed by the resazurin assay at 24, 48 and 72 hours. The cells were plated at a cell density of $0.5x10^6$ cells/mL and the resazurin was added to a final concentration of 10 µg/mL (Sigma-Aldrich) in 24 hours intervals during 72 hours. The optical density was afterwards measured at 570-600 nm using a Synergy™ HT Multi-Mode Microplate Reader, BioTek Instruments, with the metabolic activity calculated as a percentage and compared to control cells.

Cell death assessment

Cell death was evaluated by flow cytometry (FC), using the Annexin-V (AV) and 7-amino-actinomycin D (7-AAD) double staining, and May-Grünwald-Giemsa staining. For this analysis, KG-1 cells were treated with 150 nM and 250 nM of GA and 50 µM and 100 µM of

SLB; THP-1 cells were treated with 150 nM and 250 nM of GA and 75 μM and 100 μM of SLB.

Both cell lines were incubated for 72 hours as recommended by the manufacturer. Cells were washed with PBS, centrifuged at 3400 rpm for 5 minutes, resuspended in 100 μL of annexin-binding buffer and incubated for 15 minutes in the absence of light with 2.5 μL of AV-APC (BioLegend) solution and 5 μL of 7-AAD (BioLegend). After incubation, cells were diluted in 300 μL of binding buffer and analyzed by FC, using a six-parameter, four-color FACSCalibur™ flow cytometer (Becton Dickinson, USA). For each assay, $0.5x10^6$ cells were used and at least 25000 events were collected by acquisition using CellQuest software (Becton Dickinson, USA). The analysis was performed using Paint-a-gate software (Becton Dickinson, USA). Results are expressed in percentage ± standard error of the mean (SEM) of five independent experiments.

For optical microscopy analysis, cells were transferred to slides, fixed, stained according to the May-Grünwald-Giemsa staining protocol and evaluated under light microscopy, using a Nikon Eclipse 80i equipped with a Nikon Digital Camera DXm 1200F for morphological analysis (amplification 500x).[22]

Activation of caspase-3 was also evaluated by FC. Augmented fluorescence indicates the presence of caspase activity within the cells, therefore determining which cells are undergoing apoptosis.[23]

Around $1x10^6$ cells were incubated for 72 hours as described previously. Cells were washed with PBS and centrifuged at 1500 rpm for 5 minutes. Then, 100 μl of Solution A (Intra cell fixation and permeabilization kit, Immunostep) were added and vortex was performed. The cells were incubated for 15 minutes in the dark, and afterwards were washed with PBS and centrifuged at 1500 rpm for 5 minutes. After decantation, 100 μl of Solution B (Intra cell fixation and permeabilization kit, Immunostep) and 7.5 μL of FICT Rabbit Anti-Active Caspase-3 Antibody (BD Pharmingen) were added. After vortex, cells were incubated for 15 minutes in the dark. Cells were then washed with PBS and centrifuged as previously, decantated and resuspended in 500 μl of PBS. Results are expressed in percentage of activated caspase-3 ± SEM of three independent experiments.

Cell cycle analysis

Cell cycle analysis was performed by FC, using PI/RNAse solution (Immunostep). For this, $1x10^6$ cells were incubated as described previously. Then, at 72 hours, cells were centrifuged at 3500 rpm for 5 minutes and then washed with PBS, resuspended in 200 µL of 70% ethanol and incubated for 45 minutes in the dark at 4°C. Afterwards, cells were washed with PBS and resuspended in 300 µL of PI/RNase solution. After 15 minutes of incubation, cells were analyzed by FC, with the equipment already described. The results were analyzed using Modfit software (Becton Dickinson, USA) and expressed by percentage ± SEM of cells at Sub-G_1, G_0/G_1, S and G_2/M of five independent experiments.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7. Kruskal-Wallis test and ordinary one-way ANOVA were used to determine the statistical significance. A significance level of $p<0.05$ was considered. Combination Index (CI) was calculated using CompuSyn software, which defines synergism (CI<1), additive effect (CI=1) and antagonism (CI>1).[24]

Results

Effect of gambogic acid (GA) in cellular metabolic activity

The effect of single and daily fractioned administration therapy of GA in the metabolic activity of KG-1 and THP-1 cell lines is presented in Figures 1A and 1B, respectively. Our results show that GA administrated in single administration reduces the cellular metabolic activity in a time, dose and cell line dependent manner. After 72h of exposure to GA, we observed a reduction in metabolic activity with all conditions administered in both cell lines, which are statistically significant with doses of 500 and 1000 nM ($p<0.001$), with an IC_{50} of 338 nM in KG-1 and 453 nM in THP-1. Also, our results show that an administration of 150 nM divided in 50 nM fractions per day of GA (daily administration) is more efficient in decreasing metabolic activity than a single administration of 150 nM in both cell lines. When exposed to single dose administration of 150 nM, KG-1 decreased their metabolic activity to 68% and THP-1 to 73%. However, when this concentration was divided in 50 nM fractions per day, the metabolic activity decreased to 31% in KG-1 ($p<0.001$) and 45% in THP-1 ($p<0.001$).

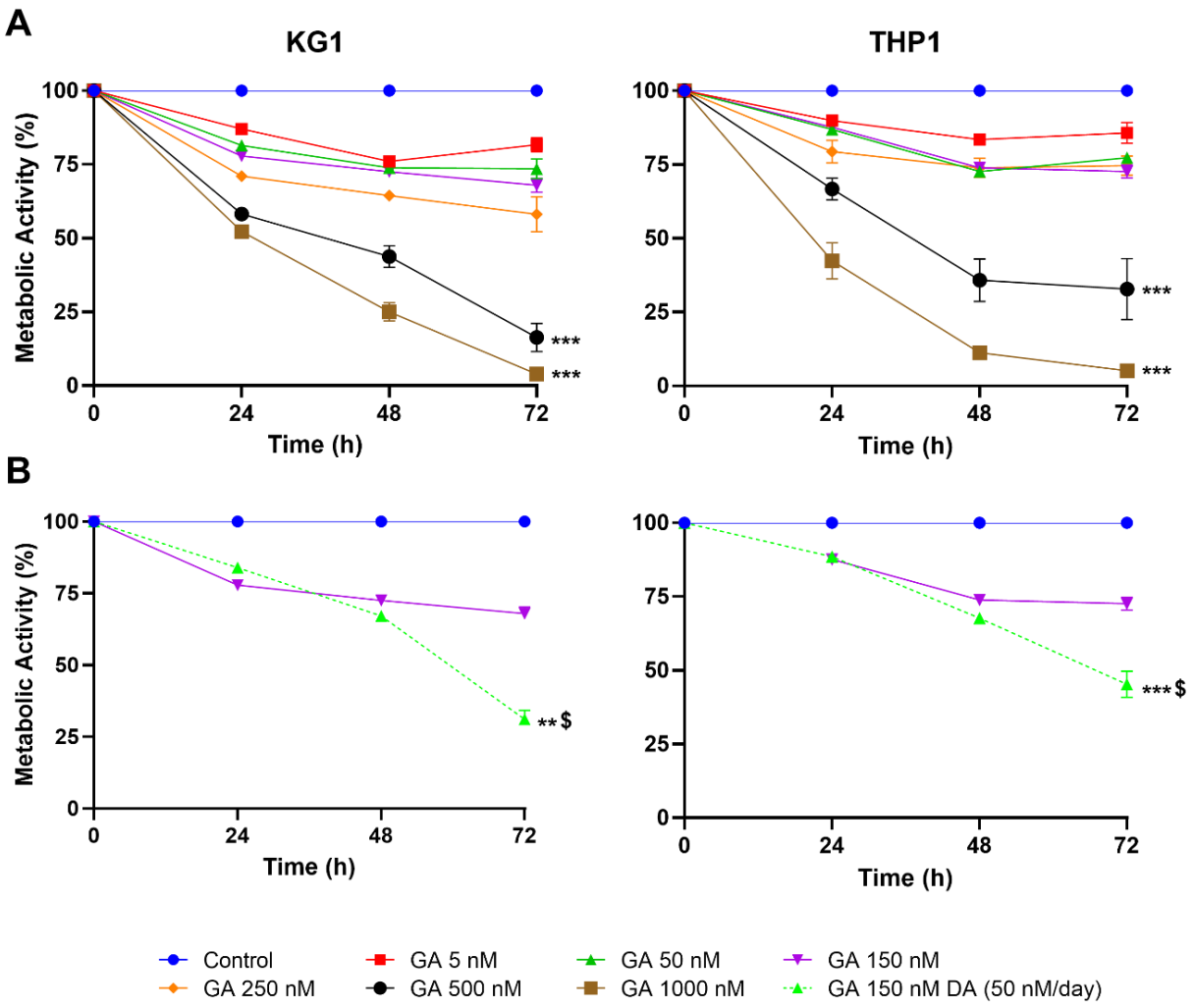

Fig. 1 – Dose-response curves of gambogic acid (GA) in single administration (A) and daily fractioned administration therapy (B) in acute myeloblastic leukemia (AML) cell lines. KG-1 and THP-1 cells were incubated for 72h in absence (control) and in presence of increasing concentrations of GA in single administration. A daily fractioned administration scheme (DA) was also tested, using 150 nM of GA divided in 50 nM fractions per day. Dose-response curves were established by the resazurin assay as described in the Material and Methods section. The results represent the mean ± SEM of 7 independent experiments. **p<0.01, ***p<0.001, when comparing different conditions with control and $^{\$}$p<0.001 when comparing daily therapy conditions with equivalent single administration conditions.

Effect of silibinin (SLB) in cellular metabolic activity

The effect of single and daily fractioned administration therapy of SLB in metabolic activity of KG-1 and THP-1 cell lines is presented in Figures 2A and 2B, respectively. Our results show that SLB administrated in single administration decreases the cellular metabolic activity in a time, dose and cell line dependent manner. After 72h of exposure to SLB, we observed a reduction in metabolic activity with all conditions used in both cell lines. In KG-1, the decrease was more notable with the doses of 100 μM ($p<0.01$) and 150 μM ($p<0.001$), being the IC_{50} of 33 μM. THP-1 presented a more accentuated decrease in metabolic activity with the doses of 75 ($p<0.01$), 100 ($p<0.01$) and 150 μM ($p<0.001$), with an IC_{50} of 75 μM. Once again, the daily administration scheme proved to be more effective in reducing the metabolic activity, but only in KG-1 cell line. After 72h, the metabolic activity of KG-1 cells reduced to 46% when in exposure to single dose administration of 50 μM, but then with administration of 45 μM of SLB divided in 15 μM fractions per day (daily administration scheme), the metabolic activity reduced to 30% ($p<0.001$). We compared the administration of 15 μM fractions per day with single administration of 50 μM because the administration of 45 μM was not possible. In THP-1, however, this difference is not significant when comparing administration of 25 μM fractions per day with single administration of 75 μM of SLB.

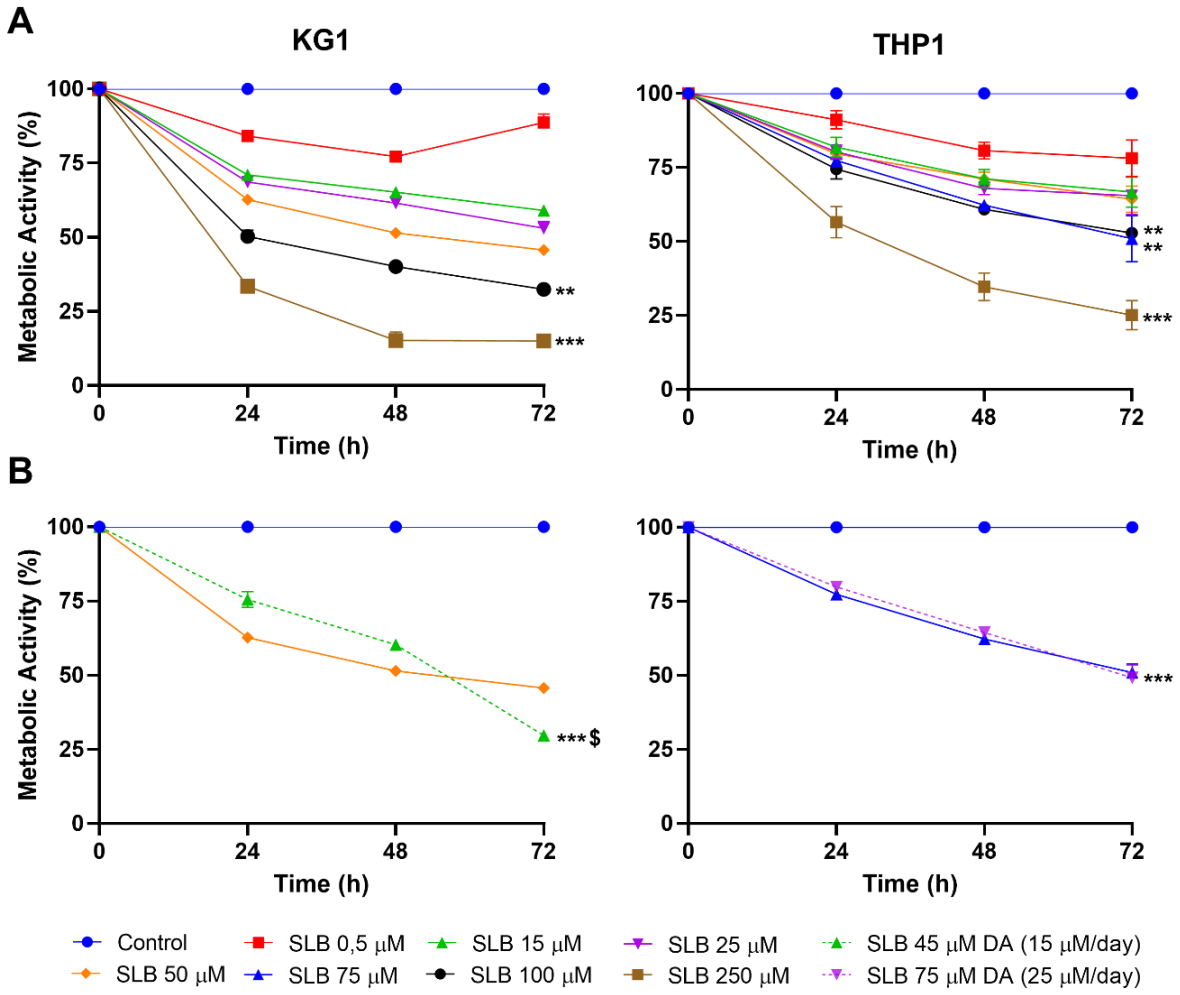

Fig. 2 – Dose-response curves of silibinin (SLB) in single administration (A) and daily fractioned administration therapy (B) in acute myeloblastic leukemia cell lines. KG-1 and THP-1 cells were incubated for 72h in absence (control) and in presence of increasing concentrations of SLB in single administration. Daily fractioned administration (DA) was also tested, using 45 µM of SLB divided in 15 µM fractions per day in KG-1 and 75 µM divided in 25 µM fractions per day in THP-1. Dose-response curves were established by the resazurin assay as described previously. The results represent the mean ± SEM of 7 independent experiments. **p<0.01, ***p<0.001, when comparing with control and $^{\$}$p<0.001 when comparing with equivalent single administration conditions.

Effect of combination therapy with gambogic acid (GA) and silibinin (SLB) in cellular metabolic activity

The effect of combination therapy of GA with SLB in cellular metabolic activity is presented in Figure 3. Our results show that a combination of 50 nM of GA and 15 µM of SLB is more effective than the same drug conditions in monotherapy, decreasing the metabolic activity of both cell lines in a time and cell line dependent manner. In fact, in KG-1 we can observe a reduction in metabolic activity after 72h to 22% with combination therapy ($p<0.001$), but when monotherapy is used, metabolic activity decreased to 74% with GA 50 nM and to 59% with SLB 15 µM. In THP-1, metabolic activity reduced to 37% with combination therapy after 72h ($p<0.001$), while a monotherapy with GA 50 nM only resulted in a reduction to 77% and to 67% with SLB 15 µM. The Combination Index (CI) for GA 50 nM associated with SLB 15 µM was 0.17 in KG-1 cells and 0.16 in THP-1, which represents a synergism (CI<1).[24]

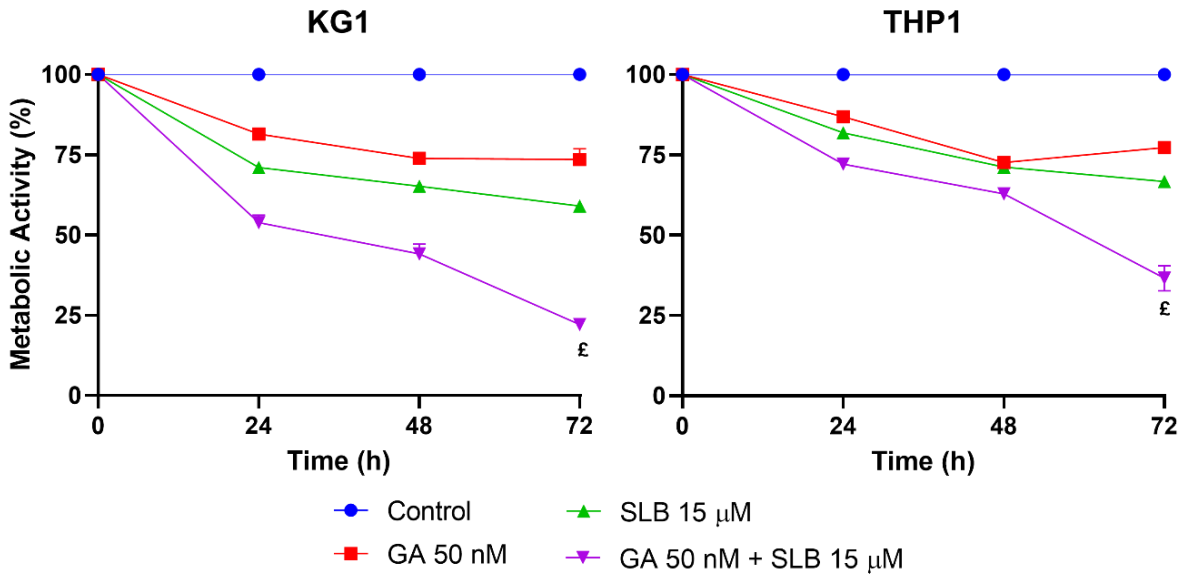

Fig. 3 – Dose-response curves of combination therapy with gambogic acid (GA) and silibinin (SLB) in acute myeloblastic leukemia cell lines. KG-1 and THP-1 cells were incubated for 72h in absence (control) and in presence of combination therapy with 50 nM of GA and 15 µM of SLB and in single administration conditions of GA 50 nM and SLB 15 µM. Dose-response curves were obtained with the resazurin assay as described previously. The results represent the mean ± SEM of 7 independent experiments. £$p<0.001$ when comparing combination therapy with single administration of 50 nM of GA and 15 µM of SLB.

Cell Death assessment

We evaluated cell death by FC, using the AV/7-AAD double staining. As represented in Figure 4, GA decreased the percentage of viable cells in both cell lines in a dose-dependent manner, being this effect statistically significant comparing the higher dose of GA (250 nM) with control (KG-1: $p<0,001$; THP-1: $p<0.01$). Cells treated with SLB showed a statistically significant decrease in viable cells of both cell lines, which is dose and cell line dependent. For KG-1 cells this statistical effect is observed with the higher dose of SLB (100 μM, $p<0.01$) and with both concentrations used in THP-1 cells ($p<0.05$ for 75 μM and $p<0.01$ for 100 μM). In KG-1 cells, we observed a statistically significant increase in percentage of early apoptotic cells, from 2% to 18% with GA 250 nM ($p<0.001$) and to 13% with SLB 100 μM ($p<0.01$), and late apoptotic/necrotic cells, from 3% to 22% with GA 250 nM ($p<0.001$) and to 11% with SLB 100 μM ($p<0.05$). An increase in necrotic cells was also observed with 250 nM of GA ($p<0.05$), from 1% to 3%. With lower drug doses, a small increase in apoptosis was observed, but not statistically significant. In THP-1, a statistically significant increase in early apoptotic cells was observed, from 3% to 11% with 250 nM of GA ($p<0.01$) and 100 μM of SLB ($p<0.01$), and in late apoptotic/necrotic cells, from 6% to 29% with GA 250 nM ($p<0.01$) and to 24% with SLB 100 μM ($p<0.01$). Regarding necrotic cells, SLB caused an increase from 2% to 22% with 75 μM ($p<0.001$) and to 13% with 100 μM ($p<0.01$).

In addition, we assessed the activation of caspase-3 in order to confirm the apoptosis induction. As represented in Figure 5, when KG-1 and THP-1 cells were treated with GA and SLB we observed an increase in activated caspase-3 levels in a dose and cell line dependent manner. In KG-1 cells, the percentage of activated caspase-3 increased from 6% to 40% with GA 250 nM ($p<0.001$) and to 30% with SLB 100 μM ($p<0.01$). In THP-1 cells, activated caspase-3 levels increased from 7% to 36% when treating with SLB 75 μM ($p<0.05$) and to 48% with 100 μM ($p<0.001$) of SLB.

Morphological analysis was also performed, as represented in Figure 6. This assay confirms our previous results, showing cellular contraction, blebbing and several vacuoles, all morphological aspects of apoptosis. Therefore, it suggests that the increase in late apoptotic/necrotic cells observed in FC studies is mainly due to apoptosis.

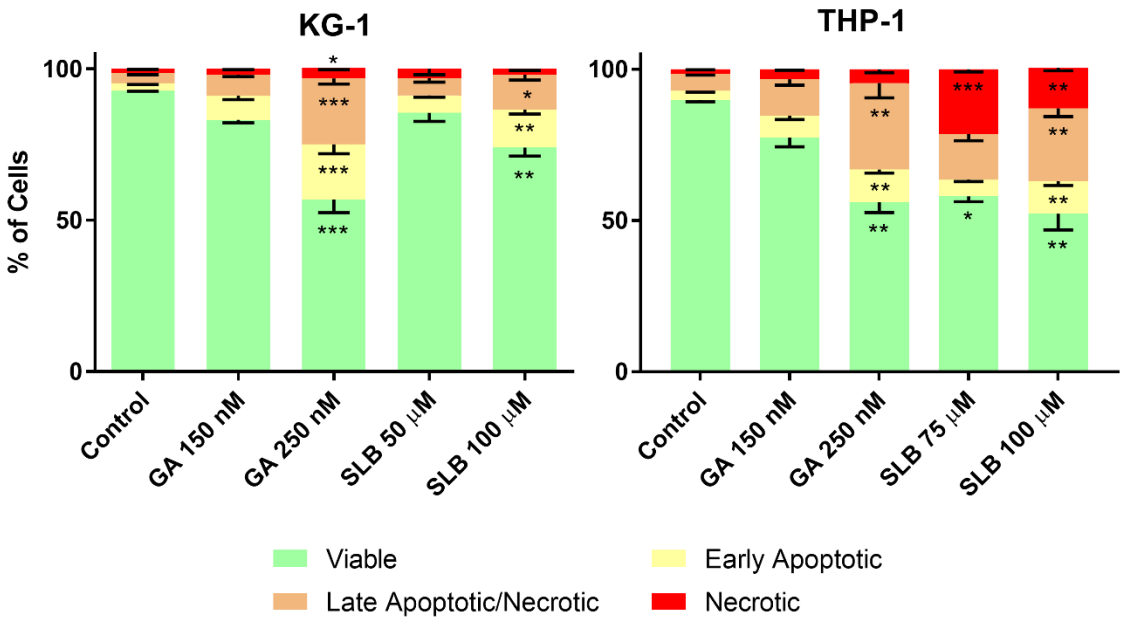

Fig. 4 – Analysis of cell death induced by gambogic acid (GA) and silibinin (SLB) in acute myeloblastic leukemia cell lines. Cell death was evaluated by FC, using the AV/7-AAD double staining, after 72h incubation with 150 nM and 250 nM of GA for KG-1 and THP-1, 75 µM and 100 µM of SLB for KG-1 and 50 µM and 100 µM of SLB for THP-1 cell line. Results are expressed as percentage (%) of cells and represent mean ± SEM of 5 independent experiments. *p<0.05; **p<0.01; ***p<0.001.

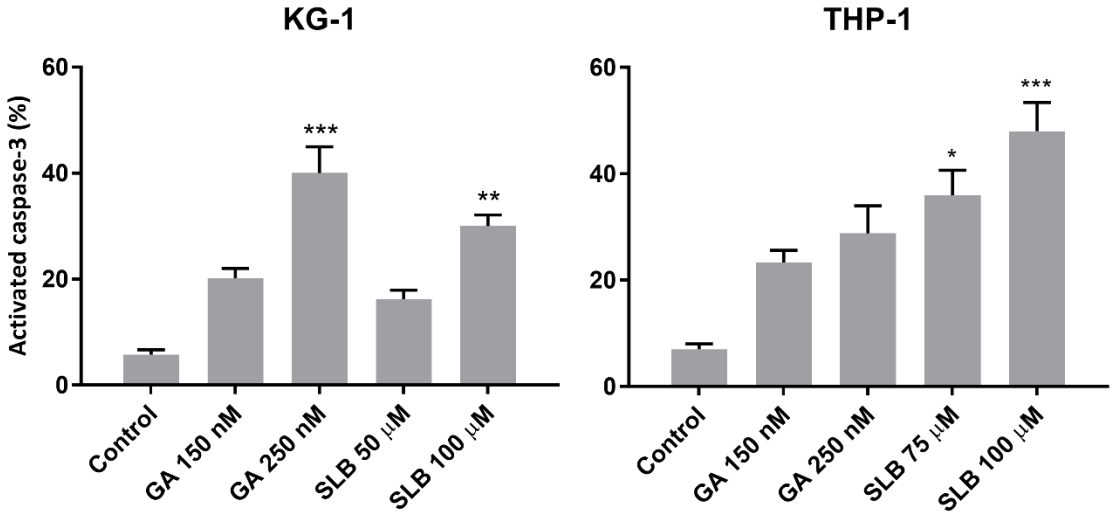

Fig. 5 – Analysis of caspase-3 activation by gambogic acid (GA) and silibinin (SLB) in acute myeloblastic leukemia cell lines. The activation of caspases was assessed by FC after 72h incubation with the drug doses represented and as described in the methods section. Results are expressed in percentage (%) of activated caspase-3 and represent the mean ± SEM of 5 independent experiments. *p<0.05; **p<0.01; ***p<0.001

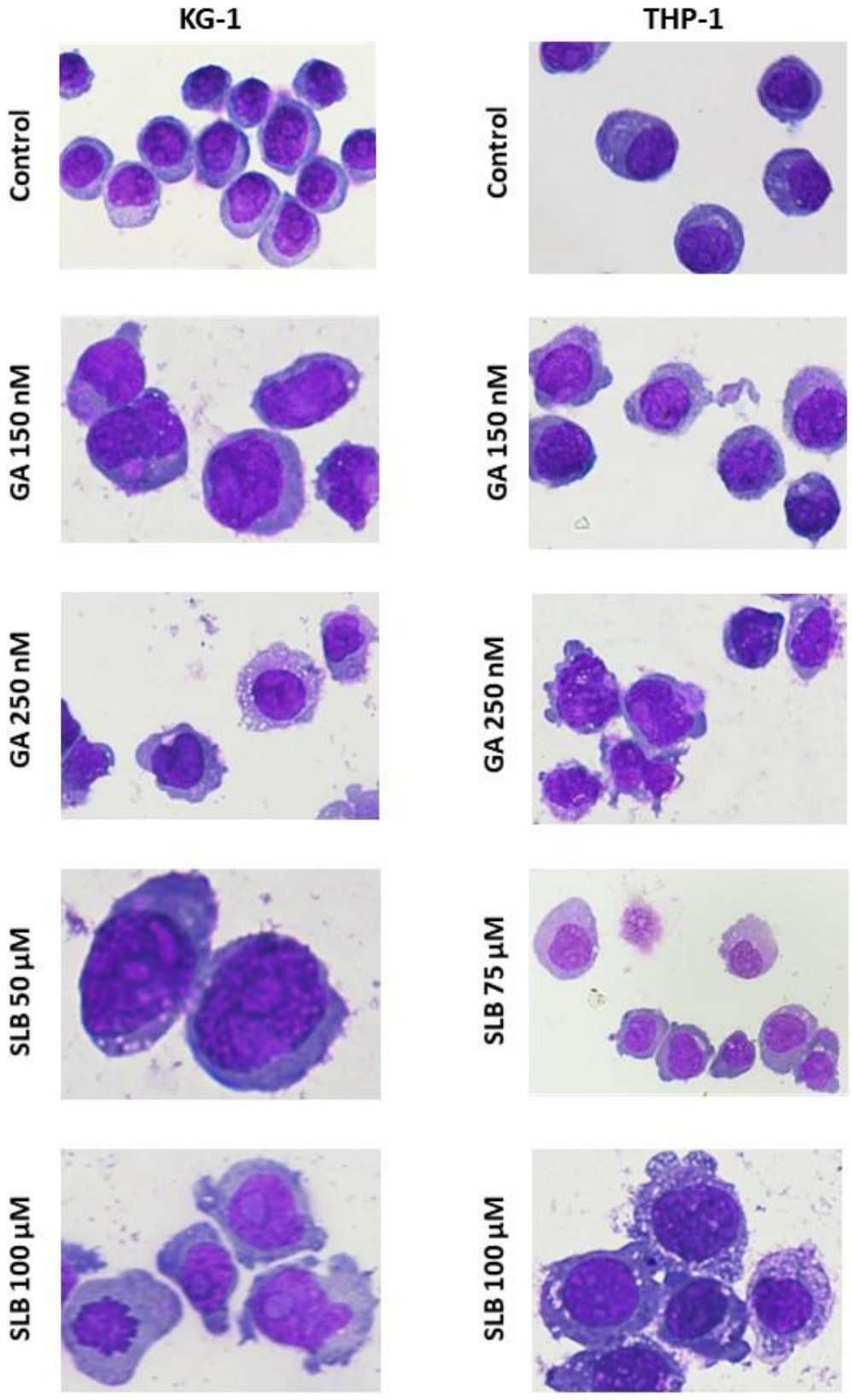

Fig. 6 – Morphological aspects of KG-1 and THP-1 after 72h of incubation with gambogic acid (GA) and silibinin (SLB). Results were obtained after staining with May-Grünwald-Giemsa and evaluation under light microscopy (amplification 500x).

Cell Cycle analysis

In Figure 7, we can see that GA has no cytostatic effect, since it does not increase significantly the percentage (%) of cells in any cell cycle phase of both cell lines. However, 250 nM of GA induced, in KG-1, an increase of cells in Sub-G_1 phase from 1% (in control) to 12%, which corresponds to an apoptotic peak ($p<0.01$). On the other hand, SLB induced in THP-1 cell cycle arrest in G_0/G_1, with both concentrations used ($p<0.01$ for 75 μM and 100 μM) and decreased the percentage of cells in S phase with 75 μM ($p<0.01$). SLB also presented an increase in percentage of THP-1 cells in Sub-G_1 phase, but not statistically significant. The mean percentage of cells in G_0/G_1 phase increased from 51% (control) to 64% when treating THP-1 cells with 75 μM of SLB and to 63% when treating with 100 μM. In KG-1, SLB had no statistically significant effect with either concentrations used.

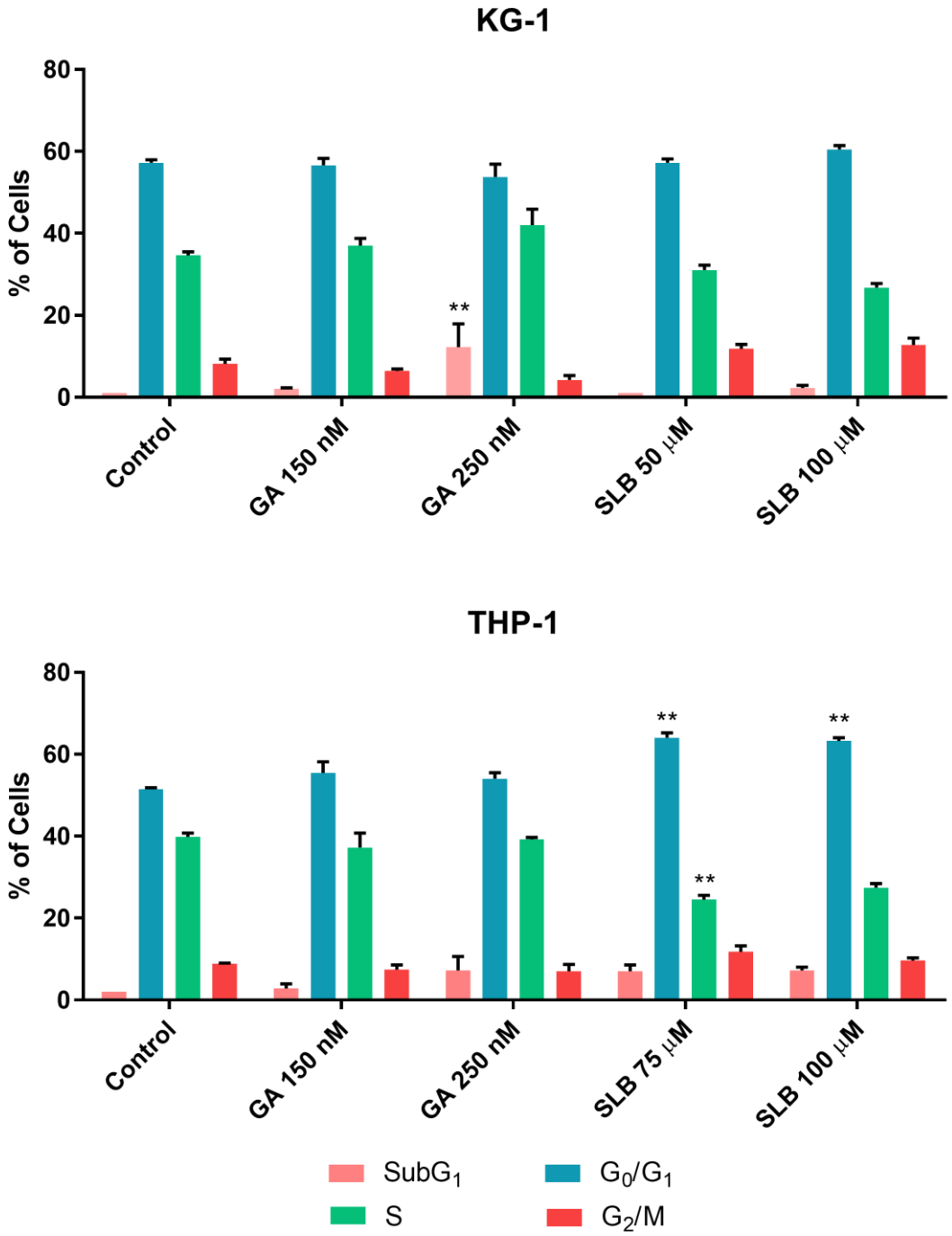

Fig. 7 – Analysis of cell cycle alterations caused by gambogic acid (GA) and silibinin (SLB) in acute myeloblastic leukemia cell lines. Cell cycle analysis was performed by FC, using PI/RNAse, after 72h incubation in presence and absence of GA and SLB in two different concentrations, as described in methods. Results are expressed in percentage (%) of cells in each cell cycle phase and represent the mean ± SEM of 5 independent experiments. **p<0.01

Discussion and Conclusion

Over the last two decades, new advances have been made in AML, namely in targeted cancer therapies as *FLT-3*[7] and *IDH* inhibitors and in immunotherapy with monoclonal antibodies[8] and chimeric antigen receptor T (CAR T) cells[8]. A *FLT-3* inhibitor, midostaurin, is approved in combination with standard cytarabine and daunorubicin induction therapy and with cytarabine for consolidation chemotherapy by the FDA for treatment of adults with newly diagnosed *FLT3*-mutated AML.[7] Ivosidenib and Enasidenib, are IDH1 and IDH2 inhibitors, respectively, that are approved by the FDA for patients with relapsed or refractory AML and an *IDH1* or *IDH2* mutations, respectively.[25,26] Gemtuzumab ozogamicin (GO), a CD33-directed antibody-drug conjugate, was reapproved in 2017 for the treatment of newly diagnosed (adults) and relapsed or refractory (aged ≥2 years) CD33+AML.[7] Currently, CAR T cell therapy is approved by FDA for treatment of pediatric and young patients with B-cell acute lymphoblastic leukemia (ALL).[27] Additionally, a study with CD7-edited ($CD7^{KO}$) CAR T cells showed remarkable results by selectively eliminating CD7-positive AML cell lines, primary leukemia blasts, and AML precursor cells *in vitro* and *in vivo*, without evident toxicity against normal myeloid cells and hematopoietic progenitors.[28] In promyelocytic leukemia (PML) therapy, new advances are represented by using retinoic acid and arsenic trioxide, suggesting that other types of acute leukemia can be controlled by selectively inducing apoptosis.[4] Further studies have shown the potential therapeutic activity of plant polyphenolic antioxidants in human malignancies.[5]

In our study, GA and SLB showed a cytotoxic effect in KG-1 and THP-1 cell lines, in a dose, time, and cell line dependent manner and significant with higher doses (500 nM and 1000 nM of GA and 75, 100 and 250 µM of SLB). These results are consistent with previous studies in AML and in other malignancies. Using similar doses (such as 500 and 1000 nM of GA) Chen *et al.* (2014) registered an IC_{50} of 0.43 ± 0.09 µM in U937 and 0.30 ± 0.02 µM in HL-60 cell lines, which are close to our results (IC_{50} of 338 nM in KG-1 and 453 nM in THP-1). Ortiz-Sánchez *et al.* (2009) tested GA in HL-60 cell line (acute promyelocytic leukemia) and registered a cytotoxic effect with a dose of 0.3 µM, by inducing apoptosis and inhibiting proliferation;[10] the dose used is only slightly lower than the most cytotoxic doses used in our study, suggesting that the difference in effect might be due to using different cell lines. Testing SLB in AML, Pesakhov *et al.* (2010) observed a mild cytotoxic effect when used alone in doses that ranged from 30 to 120 µM, with a IC_{50} of 55.54 ± 3.84 µM in KG-1 and 62.55 ± 4.44 µM in HL-60 cells, with a reduction of IC_{50} when associated to curcumin;[5] comparing to our results (IC_{50} of 33 µM in KG-1 and 75 µM in THP-1), the IC_{50} values presented are quite parallel.[5] Regarding other malignancies, a study in CML discovered that

GA efficiently induces cytotoxicity in imatinib-resistant CML cells[11] and another proved its cytotoxic effect in ATC cells.[12] Concerning SLB, Mao *et al.* (2018) demonstrated a cytotoxic effect with SLB in hepatocellular carcinoma (HCC)[19] cells and Bai *et al.* (2018) reported similar results in glioblastoma cells.[17]

Furthermore, we demonstrated that monotherapy with GA or SLB induced cell death by apoptosis in both cell lines. This result was confirmed by FC cell death analysis, caspase-3 activation studies, morphological analysis and cell cycle assessment, which revealed an increase in percentage of cells in apoptotic phase. Ortiz-Sánchez *et al.* (2009) demonstrated that GA induces apoptosis by increasing the production of reactive oxygen species, proved by FC, using a dose of 0.3 μM;[19] in our study, an increase in apoptosis was observed with the same drug concentration range (with 250 nM of GA in both cell lines). Chen *et al.* (2014) reported that lower doses, namely of 100 and 200 nM of GA, did not increase cell death by apoptosis in HL-60 and U937 lines.[9] All these findings suggest that doses equivalent or higher than 250 nM induce apoptosis, and lower do not. Regarding SLB, Pesakhov *et al.* (2010) found a slight increase in apoptosis in KG-1 and HL-60 cells when associating 60 μM of SLB to 5 μM of curcumin.[5] Curcumin is found in the Indian spice turmeric and has shown to inhibit proliferation and induce apoptosis in different types of leukemia cell lines.[5] These findings are in line with our results, since we observed an increase in apoptotic cells with 100 μM of SLB in KG-1 and THP-1, but not with 75 μM.

Many studies have linked GA and SLB to apoptosis and caspase-3 activation, namely in cells of CML,[11] bladder cancer,[13] pancreatic cancer,[15] glioblastoma,[17] gastric cancer[18] and hepatocellular carcinoma (HCC).[19] One study in CML proved that GA induced apoptosis via caspase-3 activation, through proteasome inhibition after metabolization by intracellular CYP2E1, which is highly expressed in tumor tissues but not in normal tissues, suggesting a potential tumor-specific cytotoxicity and a new way of treating cancer.[11] In bladder cancer cell lines, GA increased the processing and activation of caspases 3 and 9, enhancing apoptosis in these cells, and making GA as an attractive anti-cancer agent.[13] Caspases 3 and 9 are part of the caspase family, which is essential in cellular apoptosis; caspase 9 works as an initiating caspase of the intrinsic, or mitochondrial apoptosis pathway by activating the downstream effector caspases, such as caspase 3.[13,29] Concerning pancreatic cancer cells, GA activated caspases 3 and 7 and had an inhibitory effect in cell growth.[15] Another study reported the effects of SLB in human glioblastoma cells, showing that drug induced apoptosis through the regulation of caspase-3 and PARP-1 and concomitantly autophagy.[17] Moreover, SLB was linked to the induction of apoptosis in gastric cancer cells, by activating caspases 3 and 9, potentially through inhibition of the STAT3 pathway, which is associated

with tumor invasiveness, metastasis and prognosis.[18] Finally, a study in hepatocellular carcinoma (HCC) proved that SLB, in combination with sorafenib, induced apoptosis in HCC cells by decreasing the expression levels of antiapoptotic proteins, such as MCL-1 and BCL-2.[19] Having these results in account, it is possible to speculate that GA and SLB induce apoptosis, by activation of the mitochondrial apoptosis pathway, and autophagy, which meets our results.

Additionally, we demonstrated that SLB has an antiproliferative effect, by increasing the percentage of THP-1 cells in G_0/G_1 phase with doses of 75 and 100 µM. Wang *et al.* (2010) studied a dose of 60 µM of SLB in KG-1a, THP-1, HL-60 and U937 cell lines, in combination with 1,25-dihydroxyvitamin D3 (1,25-D), and reported similar results with cell cycle arrest in G_1/S phase.[16] Pesakhov *et al.* (2010) registered a strong antiproliferative effect with SLB in HL-60 and KG-1a, with doses ranging from 30 to 120 µM.[5] Wasserman *et al.* (2012) tested 30 mM of SLB in HL-60 cells and also observed an antiproliferative effect.[1] With all these findings, SLB appears to have an important cytostatic effect even with lower doses. The fact that KG-1 did not present a cell cycle arrest, contrarily to its variant KG-1a, may be due to its higher maturity, morphological, cytochemical, immunological and functional features and/or to differences in karyotype.[30] THP-1, on the other hand, is an acute monocytic leukemia cell line, and thus derived from a different myeloid lineage than KG-1, which was obtained from a patient with erythroleukemia, a subtype of AML with poor prognosis and known bad responsiveness to intensive chemotherapy.[31] These cellular characteristics might explain the lack of antiproliferative effect with SLB in KG-1. GA, however, lacked cytostatic effect, since no significant difference was observed in the number of cells in any phase of both cell lines.

With this work, we aimed to assess if a daily administration and a combination therapy with GA and SLB have the same, or stronger, cytotoxic effect than that of a single administration therapy, since this scheme could present less toxic side effects. In fact, we could not find scientific reviews assessing the therapeutic potential of these two drugs when associated. Comparing daily with single dose administration, GA showed a stronger cytotoxic effect in both cell lines; with SLB, however, we only observed an increased effect in KG-1 cells, being the results for single and daily administration similar in THP-1. On the other hand, we were able to demonstrate that a combination with GA and SLB had a synergic effect, being more efficacious than the administration of each drug alone, which might be due to an accentuation of the apoptotic effect of each drug by different pathways as discussed, associated to the cytostatic effect presented by SLB. Therefore, these results seem to meet our major goal.

It is known that flow cytometry is the choice technique to assess cell death, cell cycle and caspase-3 activation. We aim to complete our work by evaluating these parameters by FC testing daily administration and combination of GA with SLB. Furthermore, some authors described that GA and SLB decrease the expression levels of antiapoptotic proteins, such as MCL-1 and BCL-2,[9,19] so it could also be interesting to evaluate the expression of these proteins by FC in AML cell lines in exposure to GA and SLB. Nevertheless, the safety of these drugs as treatment for AML is still not clear and further investigations are still needed, such as performing cell cultures from AML patient samples and assays with *in vivo* animal models.

Concluding, we demonstrated that Gambogic Acid and Silibinin may bring innovation to the treatment of AML, depending on cellular characteristics and/or drug administration scheme. Both drugs proved an *in vitro* anticancer activity, mainly through induction of apoptosis, and SLB also showed an important cytostatic effect.

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