Study of DNA topoisomerases inhibition by new compounds and the regulation of these enzymes during the development of etoposide resistance in acute leukemia cell lines

Michelle X. G. Pereira[1], Amanda Sutter de Oliveira Hammes[2], Flavia C. Vasconcelos[1], Camila D. B. Müller[3], Ernesto Raúl Caffarena[2], Paulo R. R. Costa4, Raquel C. Maia[1]

1Laboratório de Hemato-Oncologia Celular e Molecular, Programa de Pesquisa em Hemato-Oncologia Molecular, INCA, RJ. 2Grupo de Biofísica e Modelagem Molecular, Programa de computação científica, PROCC, FIOCRUZ, RJ. 3Departamento de Química, PUC, RJ. 4Laboratório de Química Biorgânica, IPPN, CCS, UFRJ, RJ.

INTRODUCTION

The multidrug resistance (MDR) is an obstacle for the treatment of acute leukemias and one of the causes for the onset of refractoriness to treatment with topoisomerase inhibitors. Due to high cellular proliferation rate, tumors express 25-300 times more topoisomerases than normal cells, leading these enzymes as good targets for the development of new anticancer drugs. In this context, the development of effective new compounds on cells with resistance phenotype is necessary.

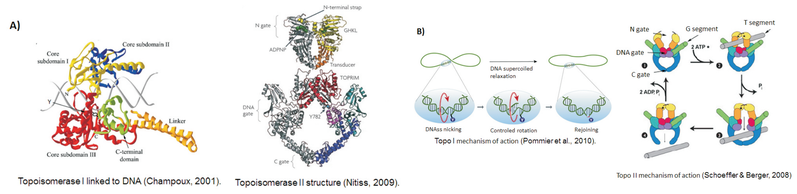


Figure 1: DNA topoisomerases scheme. A) Structure of topoisomerase I (Topo I) and topoisomerase II (Topo II). B) Topo I and Topo II mechanism of action.

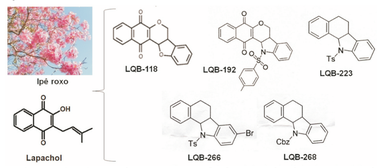


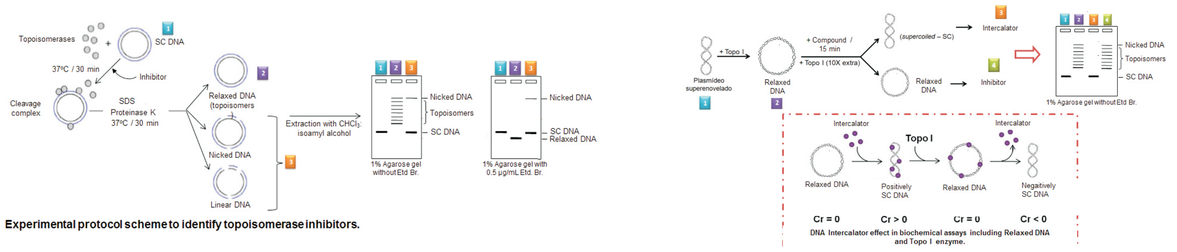
Figura 2: General structure of the synthetic compounds evaluated in this work: LQBs, initially structurally related to lapachol (LQB-118), an extracted natural naphthoquinone of *Tabebuia spp* plant. (Bignoniaceae), popularly known as "ipe".

OBJECTIVES

The aim of this work was: 1) Evaluate the potential of LQBs compounds (LQB -118, -192, -223, -266, -268 and -326) as a drug targeting human topoisomerases (hTopo I and IIα); 2) Develop acute leukemia cell lines resistant to etoposide (VP-16); 3) Investigate the effect of the most promising compounds in the induction of cell death in cell lines sensitive and resistant to VP-16.

MATERIALS E METHODS

CCRF-CEM (acute lymphoid leukemia) and U937 (acute myeloid leukemia) cell lines were cultured with increasing concentrations of VP-16 to develop the resistant cell lines CEM-R and U937-R. Cell viability was assessed by MTT assay and the IC50 was determined. MDR profiles were investigated through evaluation of P-glycoprotein (Pgp) expression and also the function of Pgp and BCRP proteins by flow cytometry. The expression of human topoisomerases was assessed by Western blot and the level of miR-548c and miR-143 were analyzed by qRT-PCR. Cell lines were treated with LQB-118 or LQB-223 and after treatment, the cell cycle profile and cell death was evaluated by flow cytometry. The mechanism of action of LQBs compounds were investigated through molecular modeling studies and by biochemical assays to evaluate inhibitory activity on hTopo I and IIα in DNA relaxation and unwinding assays.



Experimental protocol scheme to identify topoisomerase inhibitors.

RESULTS

LQB-118 and LQB-223 only inhibited hTopo IIα. The biochemical assays showed that they act as catalytic inhibitors, without intercalating in DNA. The docking of these molecules occurs with greater affinity at different sites from the binding of VP-16 to hTopo IIα and also pointed that they bind to the ATPase region of hTopo IIα. Furthermore, the theoretical model showed that the affinity for hTopo IIα is higher than hTopo IIβ. U937-R and CEM-R cell lines characterization showed changes in the expression of miR-548c and miR-143, regulators of hTopo IIα. All four lineages presented Pgp expression, however, resistant cell lines showed an increase in its expression, especially in U937-R lineage. These lineages also presented functional Pgp and BCRP proteins. LQB-118 and LQB-223 reduced viability of the resistant cell lines at similar concentrations to those used in the sensitive cell lines. Likewise, both LQBs altered the cell cycle, especially the LQB-223 that promoted stop at G2/M in all cell lines evaluated.

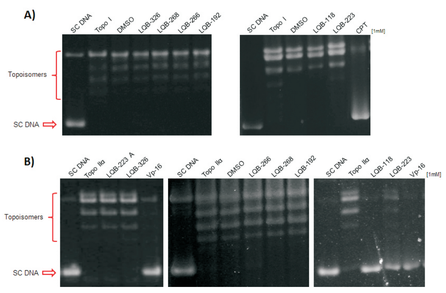


Figure 3: Relaxation DNA assay showing the effect of LQBs compounds on inhibition of human DNA topoisomerases I and IIα enzymes. (A) Assay with human Topo I e (B) Assay with human Topo IIα.

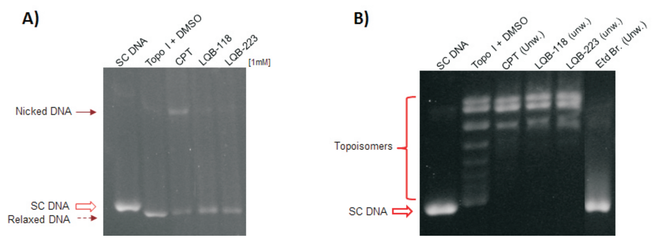
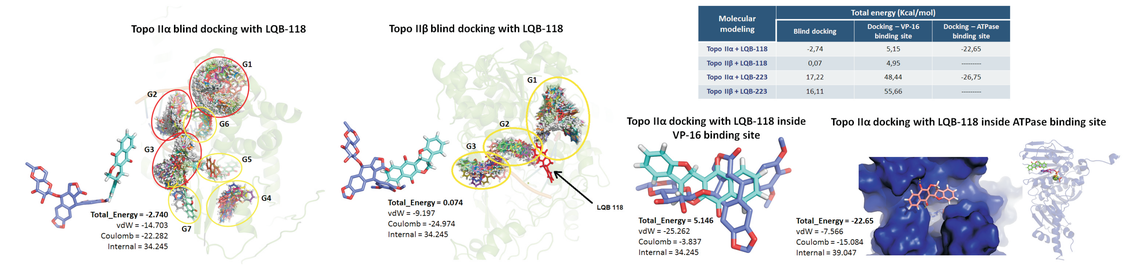


Figure 4: DNA topoisomerases mechanism of inhibition by LQB-118 and LQB-223 analysis. (A) DNA cleavage assay. (B) DNA unwinding assay.



Molecular modeling	Total energy (Kcal/mol)		
	Blind docking	Docking – VP-16 binding site	Docking – ATPase binding site
Topo IIα + LQB-118	-2,74	5,15	-22,65
Topo IIβ + LQB-118	0,07	4,95	----------
Topo IIα + LQB-223	17,22	48,44	-26,75
Topo IIβ + LQB-223	16,11	55,66	----------

Figure 5: Theorical study of LQBs-118 and -223 binding in DNA topoisomerases. VP-16 crystal in represented in blue and LBQ-118 docking in cyan. 900 conformations of each LQB were evaluated over Topoisomerases and the interactions areas were divided into different groups (G1-G7) with the prefered ones marked in red circles. The table shows the total energy values for all docking experiments.

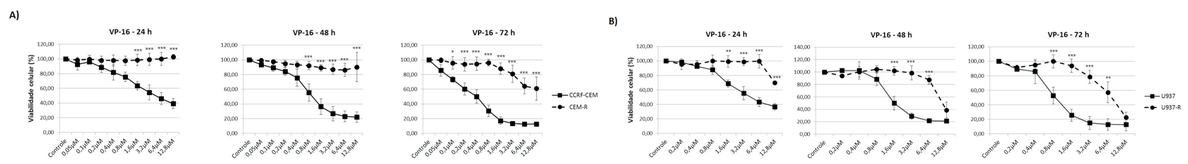
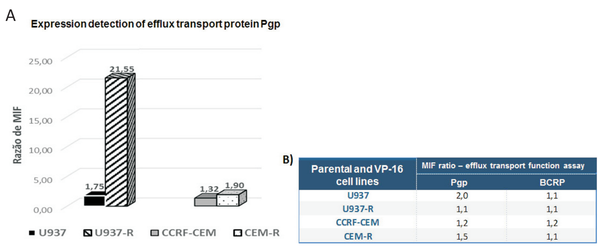


Figure 6: Characterization of Developing acute leukemia cell lines resistant to etoposide (VP-16). Cell viability after exposure to effect different VP-16 Concentrations During 24h, 48h and 72h. (A) Parental acute lymphoblastic leukemia (CCRF-CEM) and resistant (CEM-R) cell lines and (B) Parental acute myeloid leukemia (U937) and resistant (U937-R) cell line. Statistical analysis (ANOVA Followed by post-test Bonferroni) were performed comparing parental with resistant cell line (* p <0.05, ** p <0.01 and *** p <0.001).



B)

Parental and VP-16 cell lines	MIF ratio – efflux transport function assay	
	Pgp	BCRP
U937	2,0	1,1
U937-R	1,1	1,1
CCRF-CEM	1,2	1,2
CEM-R	1,5	1,1

Figure 7: Expression and function detection of proteins related to multidrug resistance profile (MDR) by flow cytometry in parental acute leukemias and resistant to etoposide (VP-16) cell lines. A) The graph represents the average of two independent experiments of Pgp and MRP expression. B) Table with the average of two independent experiments of Pgp and BCRP function. The ratio values of fluorescent intensity mean (MIF) are exemplified on the bars in the graph (A) and the table (B). MIF ≥1.1 values are considered positive.

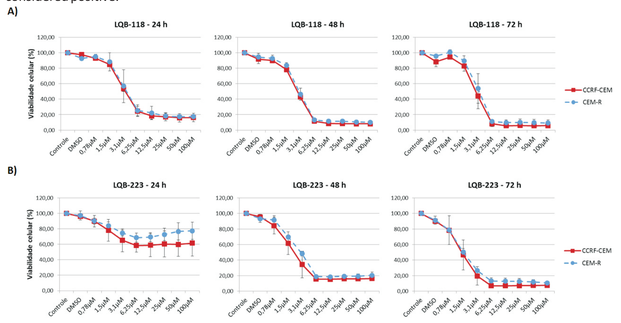


Figure 9: Cytotoxic effect of LQB-118 (A) and LQB-223 (B) compounds after 24h, 48h and 72h treatment of parental (CCRF-CEM) and VP-16 resistant (CEM-R) acute lymphocytic leukemia cell lines evaluated by MTT. Statistical analysis (ANOVA followed by Bonferroni post-test) were performed comparing parental with resistant cell line (* p <0.05; ** p <0.01 and *** p <0.001).

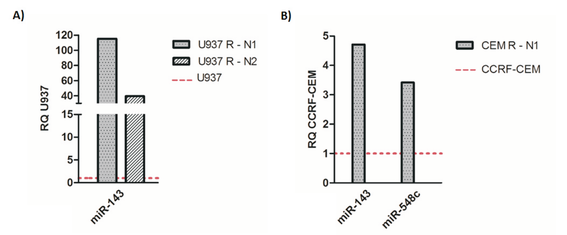


Figure 8: Expression levels evaluation of miR-143 and miR-548c on parental and VP-16 resistant acute leukemia cell lines. A) Relative expression (RQ) of miRNAs in AML cell lines: U937-R compared to U937. B) Relative expression (RQ) of microRNAs in ALL cell lines: CEM-R compared to CCRF-CEM. The expressions of microRNA were normalized with the endogenous RNU6b. The graphs show two independent experiments in AML cell lines (N1 and N2) and one experiment in strains LLA (N1) by real time PCR for each microRNA. The dotted line indicates the microRNAs expression level of the reference cell line.

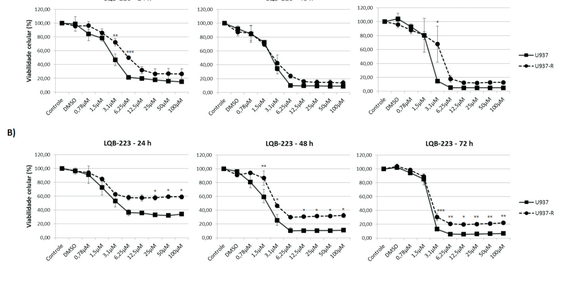


Figure 10: Cytotoxic effect of LQB-118 (A) and LQB-223 (B) compounds after 24h, 48h and 72h treatment of parental (U937) and VP-16 resistant (U937-R) acute lymphoid leukemia cell lines evaluated by MTT. Statistical analysis (ANOVA followed by Bonferroni post-test) were performed comparing parental with resistant cell line (* p <0.05; ** p <0.01 and *** p <0.001).

A)

IC50 values				
DRUGS X TIME	CCRF-CEM	CEM-R	U937	U937-R
VP-16 - 24 h	4,40	x	3,98	x
VP-16 - 48 h	0,95	x	1,62	11,20
VP-16 - 72 h	0,34	13,8	0,86	7,25
LQB-118 - 24 h	3,3	3,40	2,89	6,01
LQB-118 - 48 h	2,73	2,88	2,34	2,51
LQB-118 - 72 h	2,81	3,16	2,08	3,81
LQB-223 - 24 h	x	x	3,23	x
LQB-223 - 48 h	1,94	2,57	1,81	2,88
LQB-223 - 72 h	1,42	1,51	2,10	2,21

B)

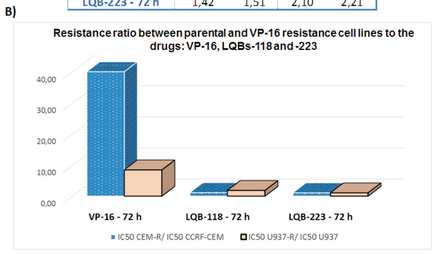


Figure 11: Evaluation of the acquisition of resistance to etoposide (VP-16) by acute leukemias cell lines and their relationship to LQBs-118 and -223 compounds. A) The table shows the IC50 values of CCRF-CEM, CEM-R, U937 and U937-R cell lines after treatment with VP-16, LQB-118 or LQB-223 for 24h, 48h and 72h. B) The graph shows the aquired resistance levels during the development of VP-16 resistant cell lines (VP-16 resistant cell lines and parental cell lines IC50 ratio).

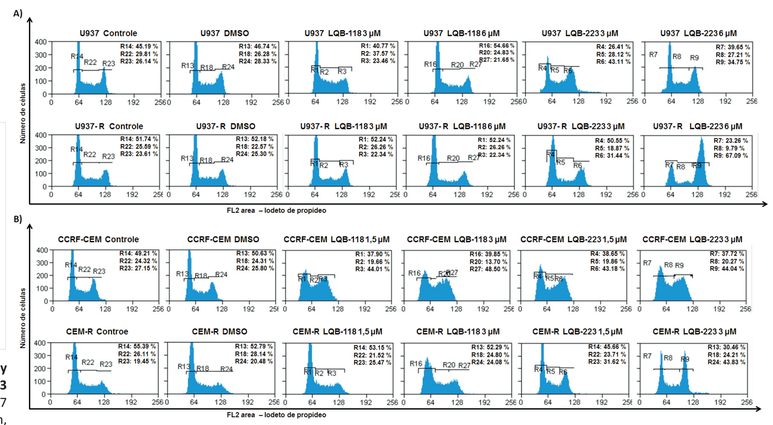


Figure 12: LQBs-118 and -223 effect on cell cycle of acute leukemias cell lines. A) U937 and U937-R treated with LQBs-118 and -223 for 24h; B) CCRF-CEM and CEM-R treated with LQBs-118 and -223 for 48h.

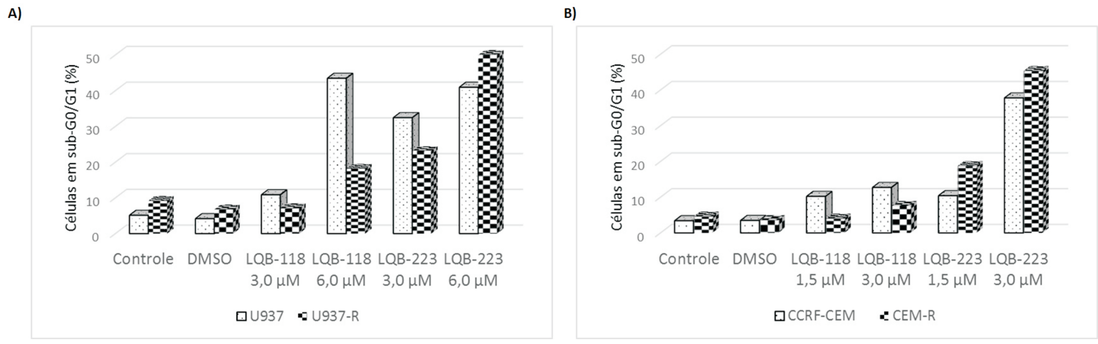


Figure 13: LQBs-118 and-223 effect on cell death induction in parental and VP-16 resistant acute leukemias cell lines. DNA fragmentation induction was Evaluated by flow cytometry in A) U937 and U937-R AML cell lines, Following 24h exposure to LQBs and in B) CCRF-CEM and CEM-R ALL cell lines, Following 48h exposure to LQBs.

CONCLUSION

Our results suggest that LQB-118 and LQB-223 are potential antitumor compounds for the treatment of acute leukemias.

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