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ARTICLE

Rubrolide analogues and their derived lactams as potential anticancer agents‡

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U. A. Pereira[a], T. A. Moreira[a], L. C. A. Barbosa[a,b]*, C. R. A. Maltha[a], I. S. Bomfim[c], S. S. Maranhão[c], M. O. Moraes[c], C. Pessoa[c,d] and F. W. A. Barros-Nepomuceno[e]

Seven β-aryl-substituted γ-alkylidene-γ-lactones analogues of rubrolides were synthesized from mucochloric acid and converted into their corresponding γ-hydroxy-γ-lactams (76-85%) by reaction with isobutylamine and propylamine. Further dehydration of the γ-hydroxy-γ-lactams led to the corresponding (*Z*)- and (*E*)-γ-alkylidene-γ-lactams (23-45%). All compounds were fully characterized by spectroscopic methods. These 14 compounds, together with 32 other rubrolide analogues were assayed against four human tumor cell lines (HL-60, leukaemia; HCT-116, colon; SF-295, central nervous system; and OVCAR-8, ovarian). Of the 46 compounds assayed, 7 caused a large reduction in cell viability (%RCV > 80%) in the test cell lines and the most active compounds had halogen substituents on the aromatic ring. Compounds **10a** and **14i** were the most active (RC_{50} = 3.00 and 3.58 μM, respectively) against HL-60 and were not cytotoxic to L929 normal cells at the concentrations tested (RC_{50} > 50 μM). To further understand the mechanism underlying the cytotoxicity of **10a** and **14i**, studies involving DNA fragmentation, cell cycle analysis, phosphatidyl serine externalization and mitochondrial depolarization were performed in HL-60 cells, using doxorubicin as a positive control. The results indicated that the cytotoxicity of **10a** and **14i** involved the induction of cell death by apoptosis. The cell cycle analysis showed that **14i** caused the accumulation of cells in G0/G1 phase at 2.5 and 5 μM.

Introduction

Transformation of a single cell into a tumorigenic phenotype is defined as cancer, where the balance between cell proliferation and cell death is disrupted. As a result of cancer, uncontrolled growth occurs, with potential cell metastasis.[1] Cancer is the leading cause of death in modern society. The International Agency for Research on Cancer estimated 14.1 million new cancer cases and 8.2 million deaths from cancers occurred in 2012,[2] which makes it a major life-threatening disease. Although enormous efforts have been dedicated to the development of new drugs for cancer treatment, there is still an urgent need to find better cures for this health problem.

For the development of more potent drugs against cancer, natural products have served as important chemical prototypes for the discovery of new molecules. Since the use of plant and microbial secondary metabolites has aided in doubling our life span in the 20th century, these compounds continue to be the most promising source of drug leads, especially in the anticancer field.[3,4]

According to Newman and Cragg,[5] over the time frame from around the 1950s to 2010, the utility of natural products as sources of novel structures is still alive and well. Of the 175 small molecules approved for use as anticancer agents, 48.6% are either natural products or directly derived therefrom.

Rubrolides are a class of γ-alkylidene-γ-lactones isolated from ascidian species, as illustrated by rubrolides K (**1**) and M (**2**) (Fig. 1). Several rubrolides and synthetic analogues are endowed with a large array of bioactivities, including antibiotic, anti-inflammatory, cytotoxic,[6,7,8,9,10] inhibition of bacterial biofilm formation,[11,12] phytotoxicity and inhibition of photosynthesis.[13,14] Work by Ortega *et al.*[7] showed that rubrolides I-N were active against four tumour cell lines, with rubrolide M (**2**) being the most active (ED_{50} = 1.2 μg/mL) against the all tumour cell lines tested.

Other classes of natural products bearing a five or six membered heterocycle, such as the γ-hydroxy-γ-lactams (**3**) and γ-alkylidene-γ-lactams (**4**) have also received attention from the chemical community due to their biological properties, including anticancer activities. The myceliothermophins A (**5**) and F (**6**), isolated from the thermophilic fungus *Myceliophthora thermophila*, were cytotoxic in the concentration range of 0.2-1.3 μg/mL to various cancer cell lines.[15] Beside the lactams,

[a] *Department of Chemistry, Federal University of Viçosa, Viçosa, MG, Brazil*
[b] *Department of Chemistry, Universidade Federal de Minas Gerais, Av. Pres. Antônio Carlos, 6627, CEP 31270-901, Belo Horizonte, MG, Brazil*
[c] *Center for Research and Drug Development, Federal University of Ceará, Rua Coronel Nunes de Melo, 1000, CEP 60430-275, Fortaleza, CE, Brazil*
[d] *Oswaldo Cruz Foundation, Av. Santos Dumont, 5753, Torre Saúde, Sala 1303, Papicu,Fortaleza, CE, Brazil*
[e] *Institute of Health Sciences, University of International Integration of the Afro-Brazilian Lusophony, Rodovia CE 060, Km 51, CEP 62785-000, Acarape, CE, Brazil*
lcab@ufmg.br; Tel.: +55 31 3899 3068; fax: +55 31 3899 3065

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some pyridazin-3(2*H*)-ones have also been highlighted as promising

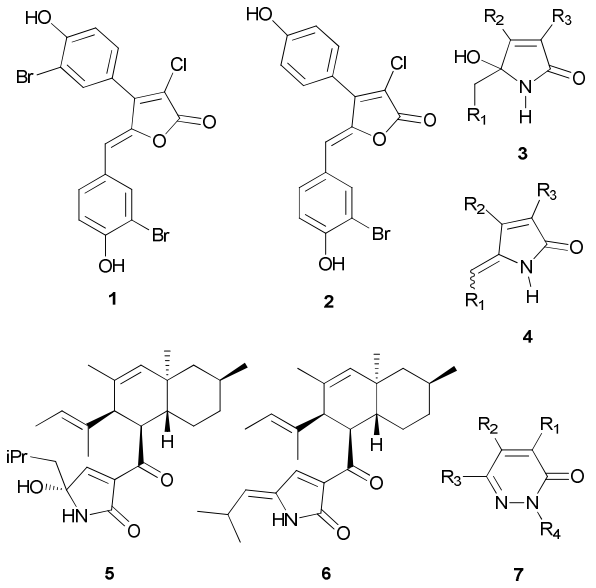


Fig. 1 Structures of rubrolide K (**1**), rubrolide M (**2**), γ-hydroxy-γ-lactams (**3**) and γ-alkylidene-γ-lactams (**4**), myceliothermophins A (**5**) and F (**6**) and pyridazin3(2*H*)-ones (**7**).

lead structures for the development of new cancer treatment agents.[16]

Accordingly and in line with the continuous effort from our group in the search of bioactive molecules with anticancer activity,[17,18,19,20] in the present work, a series of rubrolide analogues and their corresponding lactams, totalling 14 compounds, were synthesized.

These new compounds together with 32 other compounds previously synthesized,[11,12] including lactones as rubrolide analogues and their derived lactams, which had not been previously analysed for their anticancer properties, were evaluated in the present study for their cytotoxic potential against different human cancer cell lines. Also, the antitumor effects of the most active derivatives were assessed using the HL-60 cell line. Therefore, studies involving DNA fragmentation, cell cycle analysis, phosphatidyl serine (PS) externalization and mitochondrial depolarization were performed with the objective of understanding their mechanism of action.

Results and discussion

Synthesis

The lactone 3,4-dichlorofuranone **9** was prepared from commercially available mucochloric acid (**8**), with a yield of 88%. A subsequent step involved an aldol condensation between **9** and aromatic aldehydes in the presence of *tert*-butyldimethylsilyltrifluoromethanesulfonate (TBDMSOTf) and diisopropylethylamine (DIPEA), followed by treatment of the silyl ether generated in situ with DBU. Such reaction afforded the rubrolide analogues (**10a**-**10h**), stereoselectively formed in yields ranging from 10 to 58%. All compounds were fully characterized by detailed IR, NMR, and MS analyses. ^{1}H NMR of the all products showed signals related to aromatic H ranging from 6.5-8.0 ppm. Although some products were obtained in low yields, no effort to optimize the reaction conditions was made, since at this stage, we focused our attention in obtaining the final products for biological evaluation. However, from these reactions, we observed that lower yields were obtained with benzaldehydes substituted with electron-withdrawing groups, while those bearing electron-donating groups led to higher yields of the desired products. The low yield in such reactions were due in part to a rearrangement that occurred in case of aldehydes bearing electron-withdrawing groups, resulting in cyclopentenediones in large amounts.[21] According to previous studies, the compounds **10a**-**10h** have the exocyclic double bond *Z* with stereochemistry. These results are due to the steric hindrance of the β substituent in the lactone ring, as observed in the synthesis of other γ-alkylidenebutenolides.[22] While this is a possible explanation, other factors can also be involved since preferential *Z* stereochemistry in the products have also been reported for similar structures without β substituent.[23]

In a further step, lactones **10a** and **10b** were treated with excess amine to produce the corresponding γ-hydroxy-γ-lactams **11a** and **11b**, in good yields (70-84%).[24] An important feature of these compounds is the presence of a hydroxyl, which was confirmed by absorptions at 3306 and 3290 cm^{-1}, respectively for **11a** and **11b** in the IR spectrum. The hydroxylactams **11** were then dehydrated with PTSA under reflux affording γ-alkylidene-γ-lactams. As revealed by TLC analysis of the reaction mixture, two products were always formed. After purification by column chromatography, these were identified as the *Z* and *E* isomers. The stereochemistry of the exocyclic double bond was confirmed by NOE experiments, where decoupling of H-6 caused enhancements at H-7/H-8/H-9/H-10 absorptions, in the case of compound **13a**. Such results confirm that such hydrogen atoms are in close proximity in space, as expected for the isomer *E*. In general, the *Z*-compounds were obtained in better yields (56-71%) than the *E*-form (27-36%).

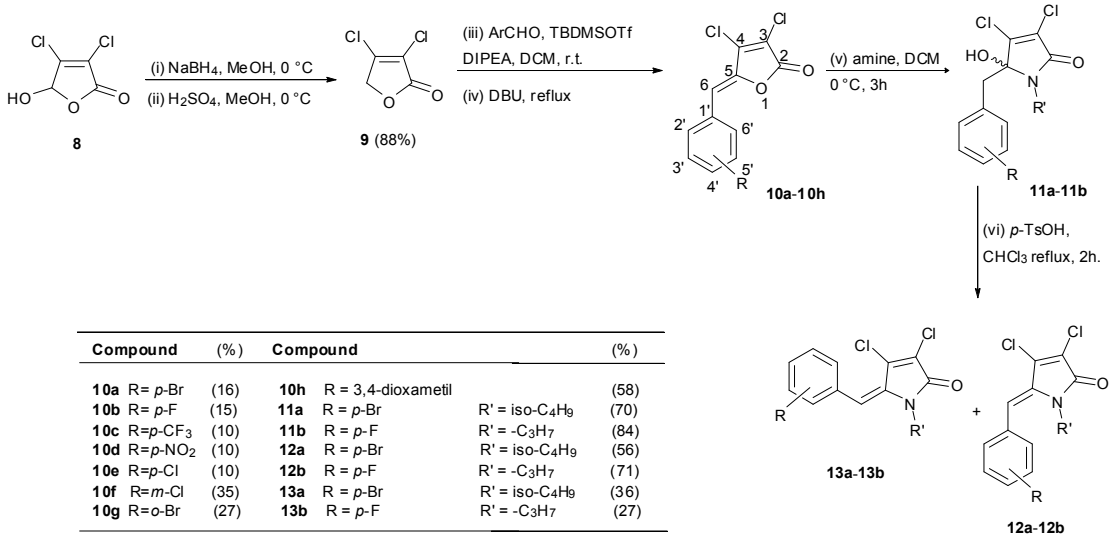
Previous work in this area led us to prepare compounds **14**-**18** (Fig. 2), which were shown to be able to inhibit bacterial biofilm formation.[12,13] For the present investigation, the synthesis was repeated to produce rubrolide analogues (**14a**-**14d** and **14f**-**14k**) and their corresponding γ-hydroxy-γ-lactams (**15a**-**15e**) and γ-alkylidene-γ-lactams (**16a**-**16e** and **17a**-**17e**) and pyridazin-3(2*H*)-ones (**18f**, **18h**-**18m**).

Biological assay

The MTT assay is widely used in cytotoxicity analysis.[25,26,27] It is a fast, sensitive and inexpensive method, described for the first time by Mosman (1983)[28] and subsequently modified by Alley *et al.* (1988)[29]. This evaluation allows one to easily determine the cytotoxicity of a particular compound, but it does not provide any insight into the mechanism of action.[30]

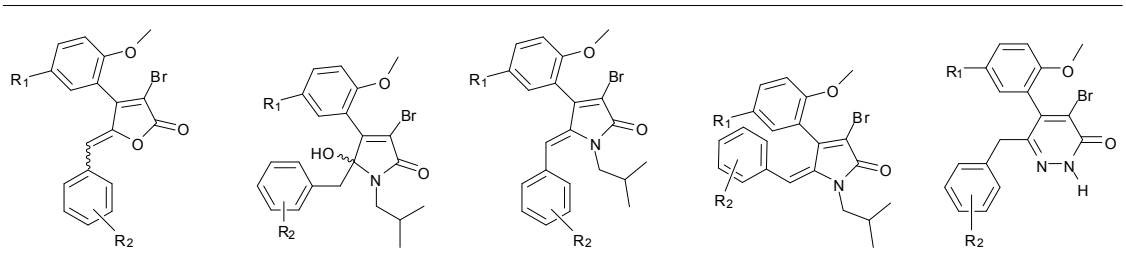
To carry out a preliminary structure-activity relationship study, the cytotoxicity of compounds mentioned in Scheme 1 and Fig. 2 were evaluated against three human tumour cell lines (HCT-116, colon; SF-295, central nervous system; and OVCAR-8, ovarian) at 25 μg/mL with 72h of incubation, using the MTT assay.

The results of antitumor activity for the 45 compounds tested are presented in Table 1 as percentage reduction in cell viability (%RCV). Compound **12b** was not evaluated due to its limited amount in our laboratory. A large reduction in cell viability was achieved when the



Compound	(%)	Compound			(%)
10a R= *p*-Br	(16)	**10h**	R = 3,4-dioxametil		(58)
10b R= *p*-F	(15)	**11a**	R = *p*-Br	R' = iso-C_4H_9	(70)
10c R=*p*-CF_3	(10)	**11b**	R = *p*-F	R' = -C_3H_7	(84)
10d R=*p*-NO_2	(10)	**12a**	R = *p*-Br	R' = iso-C_4H_9	(56)
10e R=*p*-Cl	(10)	**12b**	R = *p*-F	R' = -C_3H_7	(71)
10f R=*m*-Cl	(35)	**13a**	R = *p*-Br	R' = iso-C_4H_9	(36)
10g R=*o*-Br	(27)	**13b**	R = *p*-F	R' = -C_3H_7	(27)

Scheme 1 Preparation of γ-alkylidene-γ-lactones (**10a**-**10h**) and their corresponding γ-hydroxy-γ-lactams (**11a**-**11b**) and γ-alkylidene-γ-lactams (**12a**-**12b** and **13a**-**13b**).



	R_1	R_2
14a	H	*p*-CF_3 (*Z*)
14b	Br	*p*-Br (*Z*)
14c	Cl	*m*-OCH_3 (*Z*)
14d	CH_3	*o*-Cl (*Z*)
14f	Cl	*p*-F (*Z*)
14g/14g'	Cl	*p*-NO_2 (*Z*+*E*)
14h	Cl	*p*-CF_3 (*Z*)
14i	Br	*p*-CF_3 (*Z*)
14j	Br	*p*-F (*Z*)
14k	Br	*o*-Cl (*Z*)

	R_1	R_2
15a	H	*p*-CF_3
15b	Br	*p*-Br
15c	Cl	*m*-OCH_3
15d	CH_3	*o*-Cl
15e	Br	*m*-Cl

	R_1	R_2
16a	H	*p*-CF_3
16b	Br	*p*-Br
16c	Cl	*m*-OCH_3
16d	CH_3	*o*-Cl
16e	Br	*m*-Cl

	R_1	R_2
17a	H	*p*-CF_3
17b	Br	*p*-Br
17c	Cl	*m*-OCH_3
17d	CH_3	*o*-Cl
17e	Br	*m*-Cl

	R_1	R_2
18f	Cl	*p*-F
18h	Cl	*p*-CF_3
18i	Br	*p*-CF_3
18j	Br	*p*-F
18k	Br	*o*-Cl
18l	Br	*m*-OCH_3
18m	Cl	*m*-NO_2

Fig. 2 Analogues of rubrolides (**14a**-**14d** and **14f-14k**) and their corresponding γ-hydroxy-γ-lactams (**15a**-**15e**), (*Z*)-γ-alkylidene-γ-lactams (**16a**-**16e**) and (*E*)-γ-alkylidene-γ-lactams (**17a**-**17e**) and pyridazin-3(2*H*)-ones (**18f**, **18h**-**18m**).

compounds had %RCV greater than 75%. Thus, for HCT-116 cells, 27 compounds were very active, whereas this number of compounds was less for OVCAR-8 cells (18) and SF-295 cells (17). Accordingly, the ones with the highest %RCV against the three test cell lines (%RCV > 80%) were selected, totalling 7 compounds, for determination of concentration able to reduce cell viability by 50% (RC_{50}) by the MTT test. Among the 7 compounds, 6 of these were lactones (**10a**, **10d**, **10g**, **14h**-**14j**), only one was a lactam (**17e**) and none of them had the pyridazin-3(2*H*)-one nucleus. These results showed that the lactones had better anticancer activity compared with lactams examined and that the pyridazinones were ineffective against the tumour cell lines used in the current study. It is also important to note that the most active compounds had halogenated substituents such as F, Cl and Br on the aromatic ring, attached at carbon 3 of the lactone or lactam ring. Moreover, only one of the 7 compounds was a γ-alkylidenelactam. Similarly, Bellina *et al.*[31] demonstrated that some aryl-halogenated-furanones, including rubrolide M, are possess anticancer activity against three tumour cell lines (NCI-

H460, lung; MCF-7, breast and SF-268, CNS), in agreement with our results.

Table 1 Percent reduction in cell viability (%RCV) caused by compounds **10a-10h**, **11a**, **11b**, **12a**, **13a**, **13b**, **14a-14d**, **14f-14k**, **15a-15e**, **16a-16e**, **17a-17e**, **18f**, **18h-18m** at a single concentration (25 μg/mL) against human tumour cells after 72 h of incubation, using MTT assay

Compound	Cell lines - %RCV*		
	HCT-116	OVCAR-8	SF-295
10a	98.27	98.99	94.12
10b	97.68	98.99	68.44
10c	76.92	98.20	92.21
10d	93.05	98.20	84.12
10e	95.81	0.00	48.84
10f	62.17	99.86	86.08
10g	97.58	93.14	92.81
10h	53.64	94.90	87.84
11a	49.89	13.42	42.06
11b	25.82	9.25	10.95
12a	97.93	54.45	48.29
13a	57.48	52.94	58.59
13b	64.83	99.07	97.74
14a	84.46	98.85	71.31
14b	97.53	98.28	75.68
14c	97.09	97.92	48.09
14d	35.84	26.18	2.02
14f	55.49	32.6	84.72
14g/14g'	92.20	53.71	82.86
14h	91.81	96.84	80.10
14i	98.22	99.50	91.06
14j	94.87	94.68	82.06
14k	74.40	57.61	49.40
15a	78.84	21.61	67.94
15b	82.39	32.10	56.18
15c	67.25	27.00	69.20
15d	78.50	44.39	63.57
15e	92.75	98.49	78.09
16a	79.09	47.91	51.01
16b	44.22	25.49	30.25
16c	38.65	42.28	25.57
16d	77.02	20.46	61.86
16e	86.98	52.15	72.36
17a	88.80	75.07	84.72
17b	97.98	90.66	53.77
17c	75.51	55.28	79.36
17d	19.23	34.80	6.91
17e	93.44	93.39	90.65
18f	14.71	42.17	18.95
18h	97.73	53.87	73.87
18i	98.03	46.18	74.92
18j	59.80	29.08	48.14
8k	38.10	27.93	47.29
18l	10.78	10.69	30.95
18m	31.84	37.42	47.34

* 1% < %RCV <50% = low activity; 50% < %RCV <75% = CVR medium activity; and 75% < %RCV < 100% = high activity.

The RC_{50} data (μg/mL) for the antitumour activity of the 7 selected compounds are presented in Table 2. In this step of the study, doxorubicin was used as positive control and the tumour cell line HL-60 (human leukaemia) was added as the tumour biological model, which is commonly used in experimental anticancer research.[32,33,34]

After 72 h of incubation, all compounds tested showed moderate cytotoxic activity against the four tumor cell lines used. It is worth noting that lactones **10a** and **14i** were the most cytotoxic, with RC_{50} < 4 μg/mL against all cell lines used. Analyzing each cell line separately, compound **14i** was the most active against the cell lines OVCAR-8, SF-295 and HCT-116, with RC_{50} values ranging from 1.8 to 3.2 μg/mL, and compound **10a** was the most active against HL-60 (0.6 μg/mL). These results are in accordance with National Cancer Institute (NCI) protocols, where compounds exhibiting IC_{50} values < 4 μg/mL are considered active.[35]

As a comparison of the cytotoxicity assay results of the rubrolide analogues presented in this work with the cytotoxicity of rubrolides reported in the literature, it was found that rubrolides M and K (Fig. 1) were also active against some of the tumor cell lines tested. The best results showed that rubrolides K and M was very active against HT-29human colon carcinoma (ED_{50}= 1.2 μg/mL) among other cancer cell lines tested.[7]

The cytotoxicity of **10a** and **14i** was also evaluated to L929 normal cells (mouse fibroblasts). The results presented in Table 2 show that the cytotoxic effects of these compounds seemed to be selective for tumour cells.

Based on these results, all subsequent experiments were conducted to understand the mechanism underlying the cytotoxicity of compounds **10a** and **14i** on the HL-60 cell line.

Initially, the morphology of untreated and treated HL-60 cells was analysed by light microscopy after 24 h of incubation. The negative control cells showed typical non-adherent morphology (Fig. 3). On the other hand, HL-60 cells treated with **10a** showed intense chromatin condensation, intranuclear vacuoles, cytoplasmic budding, reduction in cell and nuclear volumes, and chromatolysis, which became more evident for incubations at higher concentrations (Fig. 3E). Additionally, an increasing number of cells with round shape were observed, suggesting a preserved membrane, but smaller than a viable cell and with an intense staining, different from the viable ones (Fig. 3C and D), which were considered apoptotic cells. Compound **14i** also promoted these apoptotic features in HL-60 cells, but with lower intensity (Fig. 3F-H) compared to **10a**. Doxorubicin (0.5 μM) induced in the HL-60 cell line cell shrinkage, chromatin condensation and nuclear fragmentation, features of apoptosis (Fig. 3B).

The results reported above were confirmed by fluorescence microscopy. After 24 h of incubation, compounds **10a** and **14i** caused a decrease in HL-60 cell viability followed by an increase in apoptotic and necrotic cells in a concentration-dependent manner (Fig. 4). Compound **10a** significantly reduced the number of viable cells just at concentrations of 5 and 10 μM with values of 17.7 and 47.3%, respectively. Also, apoptotic cells were observed at 5 μM (24.1%) and 10 μM (46.8%) as previously described. With respect to necrosis, it was shown only at 10 μM (12.7%). Treatment with compound **14i** showed a significant reduction in cell viability at all concentrations tested. At

concentrations of 2.5, 5 and 10 µM, viable cells were reduced to 67.1, 38.8 and 11.8%, respectively.

Apoptosis started occurring significantly at low concentration as 2.5 µM (31.3%), and it was extensive at 10 µM (71.5%). Necrotic cells occurred just at 10 µM (16.7%). These data show that compounds **10a** and **14i** may have a potential lead structure to develop new drugs to overcome cancer by induction of apoptosis, necrosis or necroptosis.[36,37] On the basis of morphological results suggestive of apoptosis,

Table 2 Cytotoxic effect of the compounds synthesized in four human tumour cell lines and normal cells

Compound	RC_{50}[a] (µM)				
	OVCAR-8	SF-295	HCT-116	HL-60	L929
10a	13.68 ± 1.68	14.68 ± 9.50	8.18 ± 5.91	3.00 ± 1.04	> 50
10d	9.51 ± 1.89	29.37 ± 9.1	22.76 ± 5.63	8.98 ± 2.48	ND[c]
10g	11.64 ± 5.54	32.64 ± 7.41	32.22 ± 13.95	10.01 ± 2.23	ND
14h	7.51 ± 1.41	14.12 ± 2.02	12.66 ± 1.09	5.50 ± 1.91	ND
14i	2.74 ± 1.11	4.90 ± 1.05	2.98 ± 1.84	3.58 ± 2.36	> 50
14j	5.17 ± 0.22	15.44 ± 2.55	13.06 ± 0.55	9.80 ± 2.95	ND
17e	4.18 ± 0.15	8.03 ± 0.13	5.52 ± 0.66	3.46 ± 0.04	ND
Doxorubicin[b]	0.62 ± 0.09	0.44 ± 0.28	0.04 ± 0.03	0.02 ± 0.02	ND

[a] Data are presented as RC_{50} ± SEM for ovarian (OVCAR-60), central nervous system (SF-295), colon (HCT-116) and leukaemia (HL-60) tumour cells and normal cells (L929), obtained from at least three independent experiments performed in triplicate. [b] Doxorubicin was used as positive control. [c] Not determined

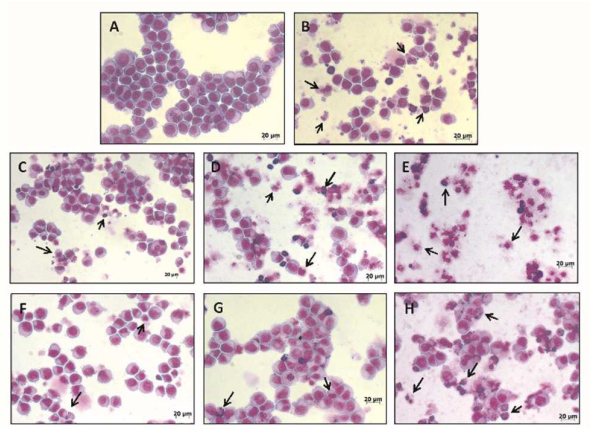


Fig. 3 Microscopic analysis (May Grünwald/Giemsa-stained) of effect of **10a** and **14i** on HL-60 cells after 24 h of incubation. Untreated cells (A) or cells treated with **10a** (2.5 µM, C; 5 µM, D; or 10 µM, E) and **14i** (2.5 µM, F; 5 µM, G; or 10 µM, H) were analysed by light microscopy (200 X). Doxorubicin (0.5 µM) was used as the positive control (B). Black arrows show reduction in cell volume, nuclear fragmentation and cellular debris.

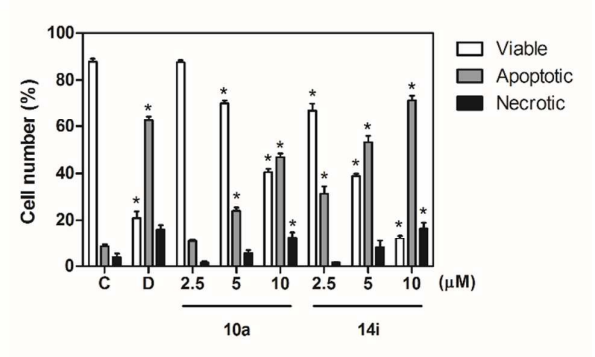


Fig. 4 Effect of **10a** and **14i** on HL-60 cell death pattern determined by acridine orange- and ethidium bromide-staining (AO/EB) after 24 h of incubation. Negative control (C) was treated with the vehicle used for diluting the test compound. Doxorubicin (0.5 µM) was used as the positive control (D). Results are expressed as mean ± standard error of mean (SEM) for three independent experiments performed in triplicate (n = 2). *p <0.05, compared to control by ANOVA followed by Newman-Keuls test.

we used flow cytometry to evaluate DNA fragmentation, cell cycle, PS externalization and mitochondrial depolarization in HL-60 cells incubated with compounds **10a** and **14i**. After 24 h, DNA fragmentation of HL-60 cells was significantly increased by the compounds in a concentration-dependent manner (Table 3).

Doxorubicin at 0.5 µM, used as a positive control, induced DNA fragmentation in 46.25% of cells, comparable with data from compound **10a** (17.50, 29.63 and 51.26%, respectively) and **14i** (15.27, 23.17 and 48.52%, respectively) at 2.5, 5 and 10 µM. Regarding cell cycle analysis (Table 3), no changes were observed after incubation with compound **10a**. On the other hand, compound **14i** caused accumulation of cells in G0/G1 phase at 2.5 and 5 µM (72.81 and 72.43%, respectively) ($p < 0.05$). Cell cycle arrest could have been related to an attempt by the cell to repair the DNA damage caused by **14i**. Since the damage appeared to be very intense, repair did not occur and apoptosis was triggered.[38] The induction of apoptosis is one of the main mechanisms that inhibit cancer growth and proliferation and is used by several antitumor agents.[39,40]

In addition, the detection of PS externalization showed that both compounds significantly induced apoptosis (Fig. 5A) at all concentrations tested. In parallel, it was found that **10a** and **14i** were also able to cause mitochondrial depolarization in a significant and concentration-dependent manner (Fig. 5A). After incubation with **10a**, the results showed that 9.5, 19.0 and 29.0% of cells displayed PS externalization and 21.7, 37.1 and 56.2 of cells showed mitochondrial depolarization at 2.5, 5 and 10 μM, respectively (Fig. 5B). Compound **14i** promoted PS externalization in 16.2, 33.3 and 48.1% of cells and mitochondrial depolarization in 35.1, 47.0 and 68.0% of cells at 2.5, 5 and 10 μM, respectively. All results presented herein suggested that compounds **10a** and **14i** induce cell death by apoptosis in HL-60 human leukaemia cells.[41]

Table 3 Effect of **10a** and **14i** on the nuclear DNA content of HL-60 cells determined by flow cytometry after 24 h of incubation.

Compound	Concentration (μM)	Cell cycle phase[b] (%)			
		Sub-G0/G1	G0/G1	S	G2/M
Control	-	6.10 ± 0.28	45.81 ± 3.16	27.64 ± 2.15	19.04 ± 1.10
Doxorubicin[a]	0.5	46.25 ± 0.81*	33.78 ± 1.10*	15.50 ± 1.45*	2.59 ± 0.21*
10a	2.5	17.50 ± 1.11*	45.29 ± 0.22	24.16 ± 1.45	7.39 ± 0.25*
	5.0	29.63 ± 4.10*	43.19 ± 2.15	14.77 ± 0.10*	4.68 ± 1.10*
	10.0	51.26 ± 2.45*	33.59 ± 1.15*	7.26 ± 2.33*	2.71 ± 0.50*
14i	2.5	15.27 ± 1.10*	72.81 ± 3.16*	13.75 ± 2.45*	5.92 ± 3.17*
	5.0	23.17 ± 4.15*	72.43 ± 1.17*	12.93 ± 0.10*	2.86 ± 1.15*
	10.0	48.52 ± 2.23*	31.64 ± 1.25*	11.81 ± 0.11*	9.12 ± 4.11*

*p <0.05, compared to control by ANOVA followed by Dunnett's test. a Doxorubicin (0.5 μM) was used as the positive control. b Results are expressed as mean ± standard error of mean (SEM) for two independent experiments performed in triplicate (n = 2).

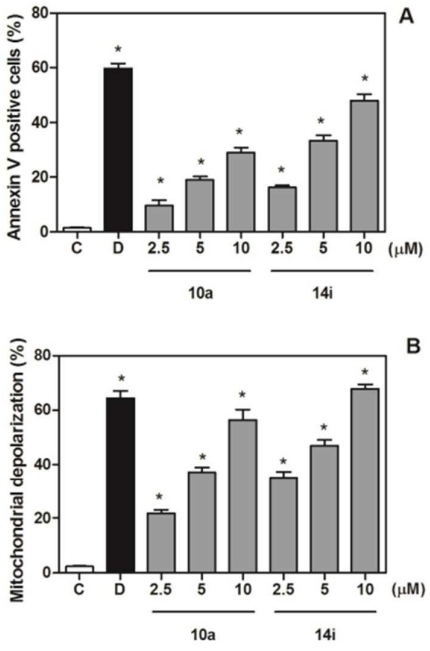


Fig. 5 Effect of **10a** and **14i** on PS externalization (Panel A) and mitochondrial depolarization (Panel B) in HL-60 cells by flow cytometry after 24 h of incubation. Negative control (C) was treated with the vehicle used for diluting the test compound. Doxorubicin (0.5 μM) was used as the positive control (D). Results are expressed as mean ± standard error of mean (SEM) for two independent experiments performed in triplicate (n = 2). *p <0.05, compared to control by ANOVA followed by Newman-Keuls test.

Conclusions

We synthesized new rubrolide analogues and converted them into the corresponding lactams and piridazin-3(2*H*)-one derivatives. All compounds tested showed moderate cytotoxic activity against the four human cancer cell lines used. In general, the presence of Br and F substituents on the aromatic ring linked to the γ position of the lactone core increased activity. Compared to lactones and lactams, the piridazin-3(2*H*)-ones did not show good activity. Moreover, compounds **10a** and **14i** displayed superior cytotoxicity against all cell lines tested. Interestingly, these compounds were not cytotoxic towards normal cells at the concentrations tested. Regarding the mechanism of action, **10a** and **14i** induced the death of HL-60 cells by induction of apoptosis, which was demonstrated by morphological analysis (volume reduction, maintenance of cell membrane integrity and nuclear fragmentation), DNA fragmentation and PS externalization. Additionally, compound **14i** caused a large increase in the number of HL-60 cells in G0/G1 phase. Accordingly, additional studies are needed to investigate the specific molecular mechanism of cell death induction and cell cycle arrest.

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Graphical Abstract