New cytotoxic rosamine derivatives selectively accumulate in the mitochondria of cancer cells

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Conventional cytotoxic anticancer drugs that target all rapidly dividing cells are nonselective in their mechanism of action, because they disrupt essential components that are crucial to both malignant and proliferating normal cells. Instead, targeting cellular functions that are distinctly different between normal and cancer cells may provide a basis for selective killing of tumor cells. One such strategy that is still largely unexplored is to utilize the relatively higher negative mitochondrial membrane potential in carcinoma cells compared with adjacent normal epithelial cells to enhance accumulation and retention of cytotoxic lipophilic cations in the former. In this study, the anticancer activities of a new class of rosamines with cyclic amine substituents and their structure–activity relationships were investigated. From an in-vitro cell growth inhibition assay, 14 of the rosamines inhibited the growth of human leukemia HL-60 cells by 50% at micromolar or lower concentrations. Derivatives containing hydrophilic substituents had less potent activity, whereas aryl substitution at the *meso* position conferred extra activity with thiofuran and *para*-iodo aryl substitutions being the most potent. In addition, both compounds were at least 10-fold more cytotoxic than rhodamine 123 against a panel of cell lines of different tissue origin and similar to rhodamine 123, exhibited more cytotoxicity against cancer cells compared with immortalized normal epithelial cells of the same organ type. In subsequent experiments, the *para*-iodo aryl substituted rosamine was found to localize exclusively within the mitochondria and induced apoptosis as the major mode of cell death. Our results suggest that these compounds offer potential for the design of mitochondria-targeting agents that either directly kill or deliver cytotoxic drugs to selectively kill cancer cells. *Anti-Cancer Drugs* 20:461–468 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Conventional chemotherapy in cancer treatment depends largely on drugs that act by interrupting DNA replication, that is, by inhibiting the synthesis or function of new nucleic materials; or by causing irreparable damage to vital nucleic acids through intercalation, alkylation, or enzymatic inhibition mechanisms. These drugs typically target rapidly dividing cells but lack selectivity for neoplastic cells, which leads to limited success in the cancer treatment. Therefore, it is important to investigate other cellular targets that are distinctly different between normal cells and cancer cells to provide a basis for selective tumor cell killing.

Mitochondria are the main energy generators, which maintain cell life and essential cell functions. Accumulating evidence show that they are also involved in diverse cellular events by being an integral part of multiple signaling cascades in regulation of metabolism, cell cycle control, development, antiviral responses, and cell death [1]. As a powerhouse, mitochondria generate energy through oxidative phosphorylation where oxidation of respiratory substrates is coupled to the synthesis of ATP under aerobic conditions. This process involves a sequence of electron transfers from respiratory substrates to oxygen, concurrent with proton translocation from the mitochondrial inner compartment to the intermembrane space through a series of respiratory chain complexes located on the inner membrane. The electrochemical proton gradient thus formed, also designated as the proton-motive force, is the driving force for ATP synthesis through the back flow of protons through the ATP synthase complex. Importantly, this mitochondrial transmembrane proton-motive force that results in a negative potential inside the mitochondrial matrix selectively accumulates lipophilic cations, which are membrane-permeable compounds with cationic characteristics [2]. High concentrations of lipophilic cations in mitochondria often result in cell death by decreasing cellular ATP production, rendering mitochondria a unique target for cellular toxicity.

Numerous studies have shown that the mitochondrial membrane potential of carcinoma cells is higher than in normal epithelial cells and that the accumulation and retention of lipophilic cations correlated with the

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mitochondrial membrane potential [3–7]. This increase in mitochondrial membrane potential in carcinoma cells, which leads to selective accumulation of toxic lipophilic cations, provides a rationale for selective chemotherapy of cancer cells. Rhodamine 123 (Rh123) was the first example of a lipophilic cation to exhibit selective antitumor activity. In in-vitro experiments, this compound markedly induced cell death in 9/9 of carcinoma cell types, whereas 6/6 of nontumorigenic epithelial cell types remained unaffected when tested at similar concentrations [8]. Other examples include the dequalinium chloride, the thiopyrylium AA1, and the thiatelluracarbocyanine iodide, which showed 10-fold to 100-fold greater inhibition of the clonal growth of carcinoma compared with control epithelial cells in culture and anticarcinoma activity in a number of whole animal tumor models [9–11]. In more recent studies, a pyridinium cation codenamed F16 was identified through a high-throughput chemical library screen as a small molecule that selectively inhibited proliferation of a variety of transformed mouse mammary epithelial cells, which had correlated increase in mitochondrial transmembrane potential [12]. An intraperitoneal injection of F16 was observed to retard the growth of A6-derived subcutaneous tumors in nude mice. In a separate example, a rhodacyanine dye known as MKT-077 was shown to significantly inhibit the growth of cancer cells *in vitro* and *in vivo*, leading to its approval as a mitochondria-targeting lipophilic cation for the treatment of carcinoma in clinical trials [13–15]. Although the clinical trials were discontinued in phase II because of a lack of efficacy at the particular approved dosage and drug regimen, the study established that MKT-077 inhibited mitochondrial function.

Despite the potential of lipophilic cations in selectively targeting cancer cells in therapeutic settings, there has not been further report from this class of compounds as new candidates for targeted cancer therapy. This study examines the cytotoxicity of a new class of lipophilic cations based on rosamine derivatives on a panel of leukemia and solid tumor cell lines, with particular attention on their structure–activity relationship. The effect of the most active derivative (compound 11) on an oral cancer cell line, HSC2 was further investigated for intracellular localization, cell cycle arrest, and the onset of apoptosis experiments.

Methods

Chemistry

The rosamine derivatives studied here with cyclic amine substituents were part of a new class of fluorescent molecules [16]. They were prepared in solution by S_NAr reactions of a relatively accessible xanthone ditriflate, followed by substitution of the triflate with cyclic amines. Addition with organolithium or Grignard reagents on the resulting vinylogous ureas followed by dehydration yielded the desired rosamine structures. Two classes of derivatives were studied in this study: compounds 1–6 have 2-methyl aryl substitution at the *meso* position and different cyclic amines at the dye core; whereas compounds 7–16 are symmetrical piperidine-substituted dye cores with a variety of *meso* substituents (Table 1). All compounds were dissolved in dimethyl sulfoxide at 10 mmol/l, aliquoted and stored at -20°C before use.

Materials

ER-Tracker Blue-White DPX, LysoTracker Blue DND-22, Rh123, and SYTOX Green were purchased from Molecular Probes, Invitrogen (Oregon, USA). Annexin V-FITC Apoptosis Detection Kit 1 was purchased from BD Biosciences (California, USA). Cell culture reagents were purchased from Gibco, Invitrogen (Auckland, New Zealand). RNase A, propidium iodide, and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, Missouri, USA).

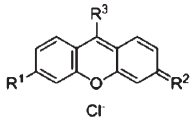
Cell cultures

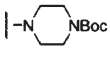
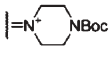
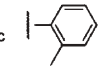
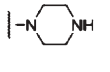
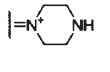
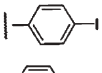
HSC2 oral cavity human squamous carcinoma cells were obtained from the Health Science Research Resources Bank (Japan). HK1 cell line is a gift from the University of Hong Kong. Both cell lines were grown in MEM medium (Invitrogen Corp., Auckland, New Zealand) supplemented with 10% fetal bovine serum. HL-60 human promyelocytic leukemia, MCF-7 breast carcinoma, and HCT-116 colon carcinoma cell lines were obtained from the American Tissue Culture Collection (Virginia, USA) and maintained in RPMI 1640 medium (Invitrogen Corp.) supplemented with 10% fetal bovine serum. OKF6, an immortalized human oral keratinocyte cell line and NP69, an immortalized human nasopharyngeal epithelial cell line were obtained from the BWH Cell Culture and the Microscopy Core (Harvard Institutes of Medicine, Massachusetts, USA) and the University of Hong Kong Culture Collection (University of Hong Kong, Hong Kong), respectively, and were maintained in keratinocyte serum-free medium supplemented with 0.1 ng/ml of epidermal growth factor, 50 μg/ml of bovine pituitary extract, and a final Ca^{2+} concentration of 0.3 mmol/l.

In-vitro proliferation assay

Approximately 4×10^3 of exponentially growing cells were seeded in each well of a 96-well plate with 50 μl of medium and were allowed to adhere overnight (for HL-60 cells, 1.5×10^4 cells/well were used). Cells were then treated with each compound at concentrations ranging from 0.01 to 10 μmol/l giving the final volume of 100 μl in each well. At the end of incubation period, 15 μl of 5.0 mg/ml MTT in phosphate-buffered saline (PBS) was added and incubated for 4 h. Medium and excessive MTT were aspirated and formazan formed was solubilized with 100 μl of dimethyl sulfoxide. Absorbance, as a measurement of viable cell number was read at 570 nm

Table 1 The structure–activity relationship and in-vitro antiproliferative activities of rosamine analogs and Rh123 in HL-60 cells



Compound	R^1	R^2	R^3	IC_{50} (μmol/l)[a]
1				0.72±0.09
2				0.76±0.03
3				0.35±0.01
4				8.3±2.2
5				0.62±0.07
6				53.3±0.3
7				3.9±1.5
8				0.82±0.04
9				0.47±0.13
10				0.10±0.04
11				0.09±0.01
12				0.66±0.17
13				0.95±0.01
14				0.25±0.22
15				2.9±1.9
16				>100
Rh123				7.8±0.6

IC_{50}, the concentration of compound which inhibits the proliferation rate by 50% as compared with control untreated cells; Rh123, rhodamine 123.
[a]Values represent the mean ± SD of three data points assessed 24 h posttreatment using the methylthiazolyldiphenyl-tetrazolium bromide assay.

with ThermoLabsystems OpsysMR microplate spectrometer (ThermoLabsystem, Chantilly, Virginia, USA).

Cellular localization

HSC2 cells grown on round glass coverslips in 12-well plate were coincubated with 100 nmol/l of compound 11 together with organelle-specific fluorescence probes. The endoplasmic reticulum was labeled with 100 nmol/l of ER-Tracker Blue-White DPX, the lysosomes were stained with 500 nmol/l of LysoTracker Blue DND-22, and the mitochondria were tracked with 100 nmol/l of Rh123, respectively, for 15–30 min of incubation at room temperature. After incubation, cells were gently rinsed with PBS to remove free dyes, and the stained cells were observed using Olympus DSU spinning disk (Olympus Optical Corp. Ltd., Tokyo, Japan) confocal microscope configured with a PlanApo 63 × oil objective and iXon EM + (Ardor Technology, South Windsor, Connecticut, USA) digital camera. Fluorescence images of *x*–*y* sections at 0.2 μm were collected sequentially using Olympus Cell software (Olympus Soft Imaging Solutions, München, Germany). Organelle-specific fluorescence probes were excited at 365 nm to illuminate ER-Tracker and LysoTracker, at 494 nm for Rh123, and at 575 nm for compound 11, respectively.

Annexin V–fluorescein isothiocyanate apoptosis analysis

HSC2 cells grown in 60-mm dishes at 50% confluency were treated with 0.5 μmol/l of compound 11. At various treatment intervals, floating cells in the medium were pooled together with the adherent cells after trypsinization and were washed twice with cold PBS. The cells were resuspended with 1 × binding buffer [0.01 mol/l Hepes/NaOH (pH 7.4), 0.14 mol/l NaCl, 2.5 mmol/l $CaCl_2$] at 1×10^6 cells/ml. A 100 μl of cell suspension was transferred to a flow cytometry tube followed by 5 μl of annexin V–fluorescein isothiocyanate (FITC) and 5 μl of 200 μg/ml propidium iodide in PBS. The cells were gently mixed and incubated for 15 min at room temperature in the dark before analyzed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) with 488 nm argon laser. The fluorescence data of 1×10^4 cells were collected with the FL1 detector with 530/30 band-pass filter to collect annexin V–FITC fluorescence, and the FL3 detector with a 630 nm long-pass filter to collect propidium iodide fluorescence.

Cell cycle analysis

HSC2 cells were treated and collected as above. Cells were then fixed in 70% ice-cold ethanol (v/v in PBS) overnight at 4°C. After fixation, the cells were washed twice in cold PBS. The pellet was then resuspended in PBS solution containing 20 μg/ml RNase A and 1 μmol/l SYTOX Green for 30 min. The cells were analyzed on a FACSCalibur flow cytometer with a 488 nm argon laser.

The DNA-SYTOX Green fluorescence of 1×10^4 cells were collected with the FL1 detector with a 530/30 bandpass filter.

Results and discussion

In-vitro antiproliferative assay

The in-vitro antiproliferative activity of compounds 1–16 against a promyelocytic leukemia cell line, HL-60, was determined using a 24 h endpoint MTT assay. Results were expressed as IC_{50} – the concentration of compound (in μmol/l), which inhibits the proliferation rate by 50% as compared with control untreated cells. Rh123, a commercially available compound with a similar structure was also tested for comparison. From the assay, compounds 1–3, 5, and 8–14 showed antitumor activity with IC_{50} values in the submicromolar range. Compound 10, which had a thiofuran and the *para*-iodo aryl substituted compound 11, showed the highest activity among the analogs, with IC_{50} values (0.10 and 0.09 μmol/l, respectively) that are more than 50 times lower than that of Rh123 (7.8 μmol/l). In contrast, compounds 4, 6, 7, 15, and 16 displayed moderate-to-poor activity from single-digit micromolar IC_{50} values to undeterminable IC_{50} up to 100 μmol/l.

The influence of the cyclic amine substituents on the antiproliferative activity of the compounds was evident from studying compounds 1–6. Regardless of the size of the ring, the derivatives containing hydrophobic cyclic amines from 5-membered pyrrolidine (compound 1) to 6-membered piperidine (compound 2) and Boc-piperazine (compound 5) exhibited moderate antiproliferative activity with IC_{50} values of 0.62–0.76 μmol/l. In contrast, the derivatives with cyclic amines that contain exposed oxygen or NH isosteres as in the case of compounds 4 and 6 had 10-fold to 70-fold higher IC_{50} of 8.3 or 53.3 μmol/l, respectively. The unsymmetrical rosamine 3, which had a combination of piperidine and morpholine substituents interestingly, had the lowest IC_{50} value among compounds 1–6, alluding to the possible importance of an ampiphilic structure with contrasting hydrophobic and hydrophilic halves.

For the effect of *meso* substitution on the antiproliferative activity of rosamines, compounds 7–16 were studied. Similar to compounds 4 and 6 mentioned above, the derivatives with hydrophilic substituents such as the phenolic 15 and the carboxylic 16 had higher IC_{50} values than the unsubstituted *meso*-aryl 9. Having an aryl subtituent at the *meso* position, whether directly (compounds 9–14) or through an alkyl spacer (compound 8), was important for the antiproliferative activity and was convincingly shown in the lower activity observed in compound 7, which had only a simple methyl substituent at the *meso* position. Among the aryl-substituted compounds, the thiofuran (compound 10) and the *para*-iodo aryl (compound 11) structures had the lowest IC_{50} values whereas 4-methoxy aryl (compound 12), mono-2-methoxy (compound 13), and di-2-methoxy (compound 14) aryl substitutions did not confer additional activity compared to the phenyl substituted compound 9.

Table 2 In-vitro antiproliferative activities of rosamine analogs in carcinoma and immortalized normal human epithelial cell types

		IC_{50} (μmol/l)[a]		
Cell line	Tissue origin	Compound 10	Compound 11	Rh123
HCT116	Colon	0.15 ± 0.06	0.39 ± 0.11	7.9 ± 1.0
MCF-7	Breast	0.27 ± 0.16	0.39 ± 0.22	5.6 ± 0.6
HSC2	Oral	0.12 ± 0.09	0.25 ± 0.12	4.5 ± 2.2
OKF6[b]	Oral	0.25 ± 0.10	0.41 ± 0.07	9.8 ± 3.5
HK1	Nasopharynx	0.09 ± 0.01	0.42 ± 0.06	5.9 ± 0.2
NP69[b]	Nasopharynx	0.33 ± 0.20	0.51 ± 0.16	6.3 ± 0.2

IC_{50}, the concentration of compound, which inhibits the proliferation rate by 50% as compared with control untreated cells; Rh123, rhodamine 123.
[a]Values represent the mean ± SD of three data points assessed 48 h posttreatment using the methylthiazolyldiphenyl-tetrazolium bromide assay.
[b]Immortalized normal human epithelial cells.

Subsequently, the in-vitro antiproliferative activity of the most active compounds, 10 and 11, were assessed against a panel of cell lines derived from human solid tumors including colon cancer, breast cancer, oral squamous cell carcinoma, and nasopharyngeal carcinoma (Table 2). A 48-h assay endpoint, which is more typical of cytotoxicity studies and which was used by the US NCI in-vitro anticancer drug screen [17], was used. The antiproliferative activity of Rh123 was also simultaneously determined for comparison. Both compounds 10 and 11 exhibited at least 10-fold lower IC_{50} values compared with Rh123. In addition, the thiofuran-substituted compound 10 consistently showed between 1.5-fold and 4-fold lower IC_{50} values across all four types of solid tumors compared with compound 11.

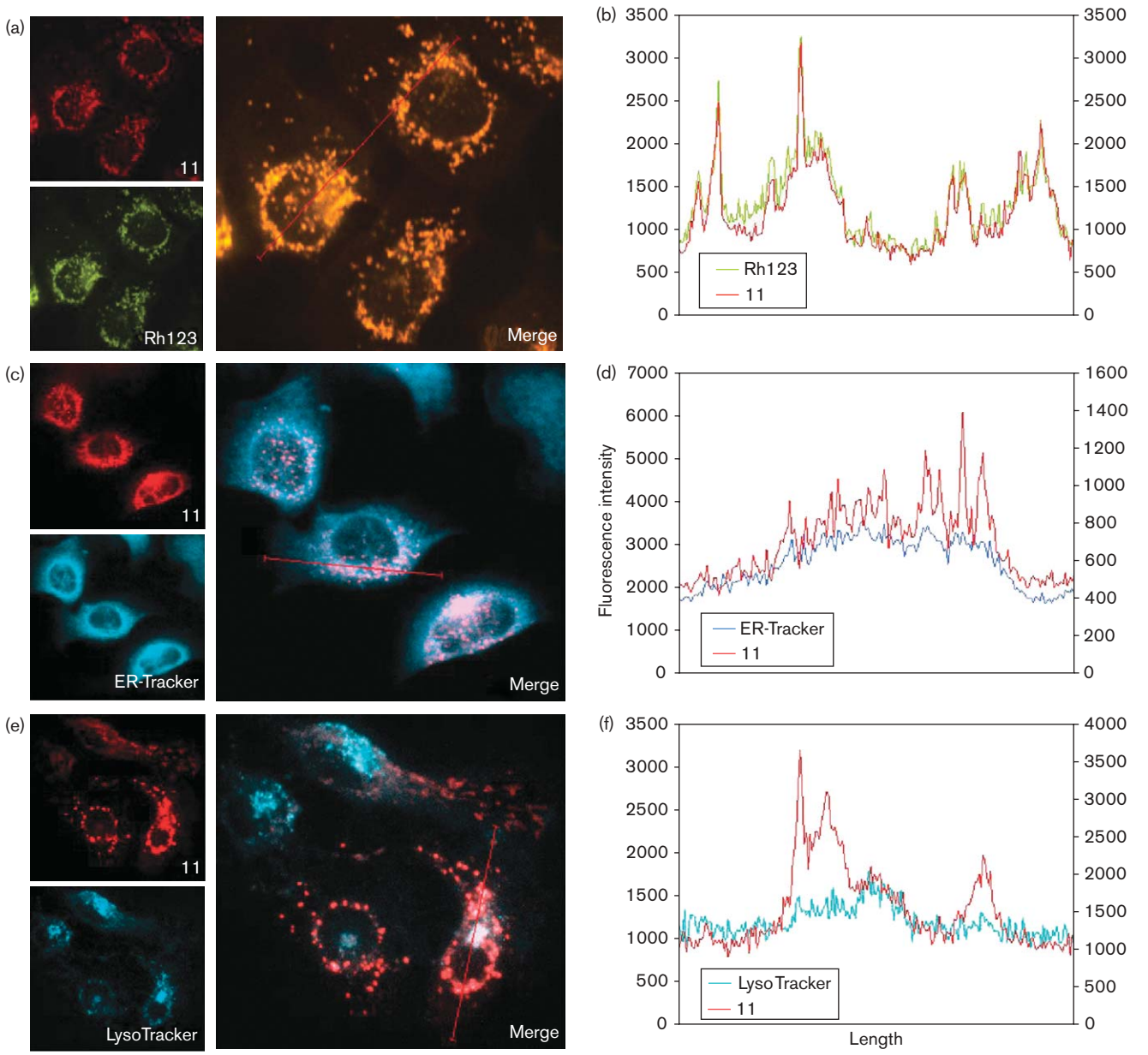
To investigate whether rosamines 10 and 11 have greater antiproliferative effects on cancer cells compared with normal cells, two immortalized epithelial cell lines from oral (OKF6) and nasopharyngeal (NP69) origins were also included in the study. Both compounds 10 and 11 were found to be more cytotoxic toward the cancer cell lines than the normal cell lines, as shown in the 1.25-fold to 3-fold higher IC_{50} values in the normal compared with the cancer cell lines. Importantly, the fold-selectivity observed for both compounds is similar to that exhibited by Rh123 in both tissue types (1.1-fold to 2.2-fold in Table 2). The relatively modest fold differences observed here between cancer and normal cell lines compared with those reported in the literature [4,8] may be because of a number of reasons, including differences in the characteristics of the cell lines studied. Direct comparison between tumor and its surrounding tissue in terms of compound accumulation and uptake can be properly studied in an in-vivo tumor model.

Cellular localization of compound 11

Although both compounds 10 and 11 have equally potent antiproliferative activity with similar IC_{50} values in the low submicromolar range, the fluorescence quantum yield of compound 10 is low, at a value that is approximately 3-fold lower compared with compound 11 (0.28 ± 0.01 vs. 0.10 ± 0.01 in ethanol) [16]. Therefore, only the intracellular localization of compound 11 in HSC2 cells was examined by confocal microscopy using dual-staining techniques (Fig. 1).

Costaining images and topographic profiles of cells containing compound 11 and a mitochondria-specific dye Rh123 revealed an almost identical overlap, suggesting that compound 11 localized particularly well in mitochondria (Fig. 1a and b). In comparison, compound

Fig. 1



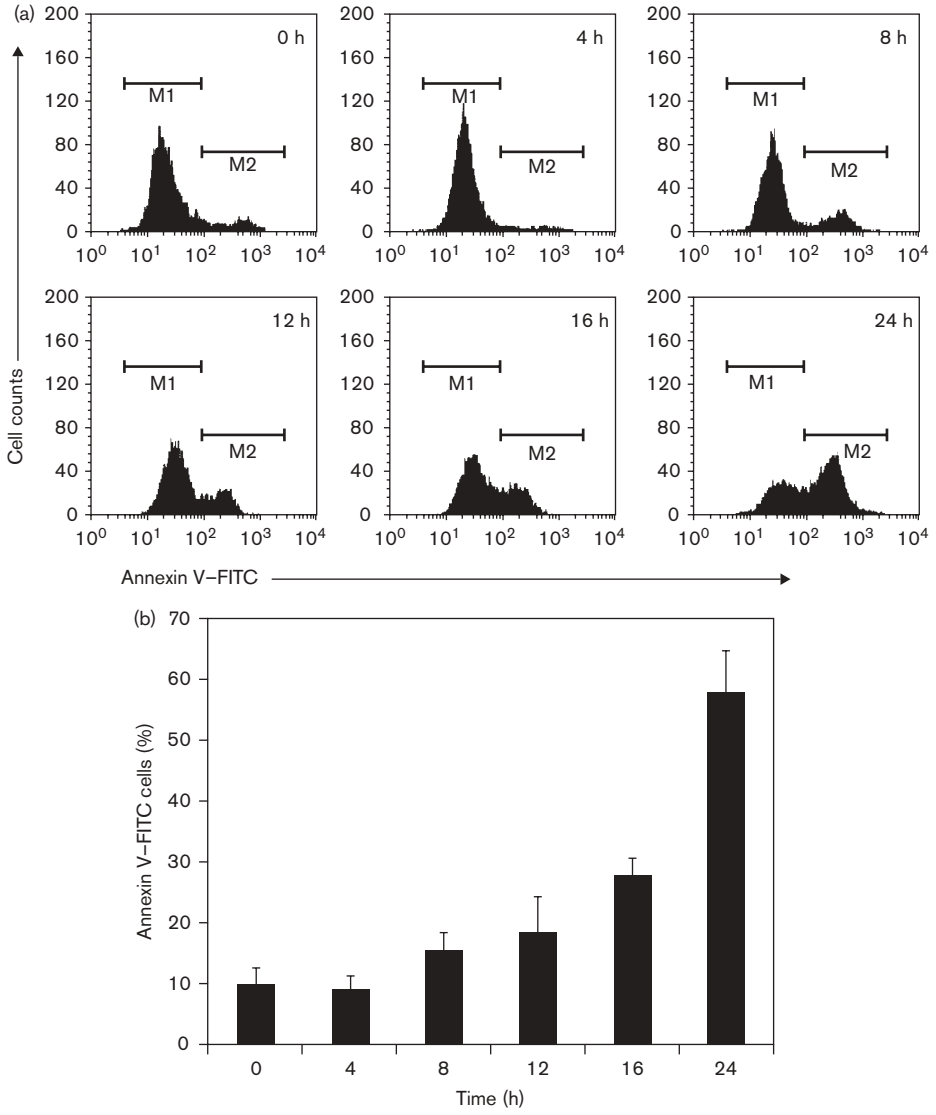
Intracellular localization of compound 11 in HSC2 cells. Confocal images (a,c,e) and fluorescence topographic profiles (b,d,f) of HSC2 cells double-stained with 100 nmol/l of compound 11 and respective organelle probes. (a,b) Mitochondria were labeled with 100 nmol/l of rhodamine 123 (Rh123) and excited at 494 nm. (c,d) Endoplasmic reticulum were labeled with 100 nmol/l ER-Tracker and excited at 365 nm. (e,f) Lysosomes were labeled with 500 nmol/l of LysoTracker and excited at 365 nm. Compound 11 was excited at 575 nm. Line segment in confocal images indicates the longitudinal transcellular axis analyzed to generate the topography fluorescence profiles. Objective magnification, $\times 63$.

11 displayed only partial colocalization with endoplasmic reticulum and lysosomes, according to the confocal images and topographic profiles of compound 11 with ER-Tracker (Fig. 1c and d) and LysoTracker (Fig. 1e and f), respectively. Staining of the cytoplasmic or nuclear membrane by compound 11 was not detected, indicating that it does not react nonspecifically with biological membranes. Furthermore, the nucleus remained free of compound 11 (dark nuclear area) signifying that this class of compounds would not be expected to directly damage DNA.

Apoptosis and cell cycle studies on compound 11

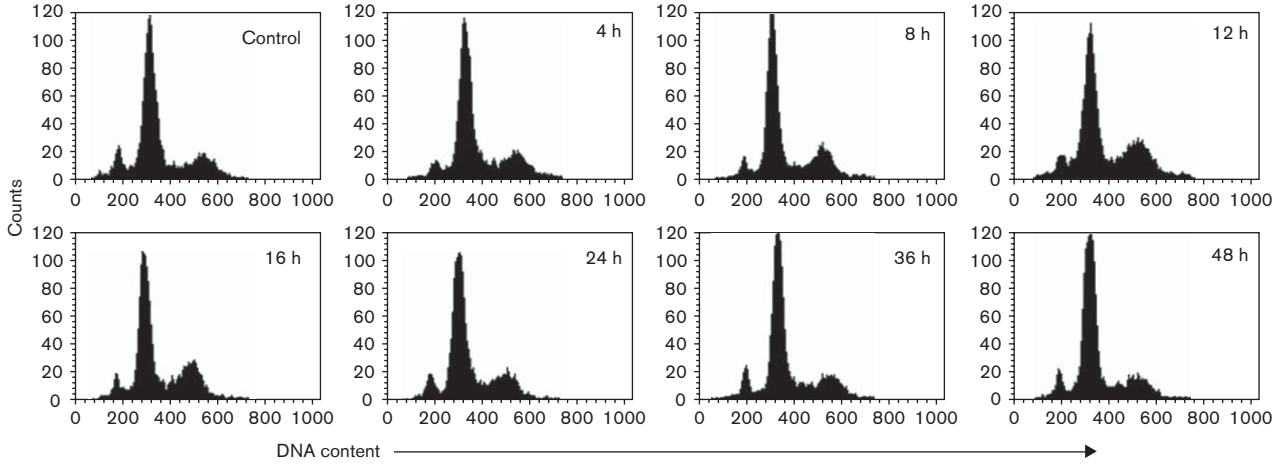
The induction of apoptosis was quantified in flow cytometry experiments measuring the externalization of membrane phosphatidylserine through annexin V–FITC staining, an event which is considered characteristic of cells undergoing apoptosis (Fig. 2). Flow cytometric analysis of HSC2 cells treated with 0.5 μmol/l of compound 11 showed the onset of apoptosis by 8 h of incubation with 16% of the cells stained positive for annexin V compared with less than 10% at 0 or 4 h timepoints. The proportion of cells undergoing apoptosis continued to increase rapidly to 58% within 24 h. However, compound 11 does not induce cell cycle arrest. The cell cycle profile of HSC2 when treated with 0.25 μmol/l of compound 11 was examined in a time course experiment. From 4 to 48 h, the cell cycle profile remained unchanged (Fig. 3), showing that the cell death

Fig. 2



(a) Representative histograms of cells and (b) mean percentage of cells with annexin V–fluorescein isothiocyanate (FITC) binding to phosphatidylserine as an indicator of apoptosis in HSC2 cells treated with 0.5 μmol/l compound 11 for different durations. M1 represents viable cell population, M2 represents apoptotic cell population.

Fig. 3



Effects on HSC2 cell cycle phase after treatment with 0.25 μmol/l of compound 11 for various time intervals and analyzed using flow cytometry.

caused by compound 11 did not occur as a result of cell cycle arrests.

Conclusion

We have shown the antiproliferative activity of a new class of rosamine derivatives with cyclic amine substituents, against a panel of cell lines from leukemia and solid tumors. A structure–activity relationship study indicated the importance of having hydrophobic substituents at the peripheral cyclic amines as well as at the *meso*-aryl groups. Among the meso-aryl structures, compounds 10 and 11 containing *para*-iodo and thiofuran *meso* substituents, respectively, had the most potent activity. In addition, compounds with amphiphilic cyclic amines, as in the case of compound 3, may also show significantly lower IC_{50} values compared with the symmetrical hydrophobic structures. Combining these observations, it would indeed be interesting in follow-on work to investigate whether a structure with piperidine and morpholine cyclic amines as well as a *para*-iodo or a thiofuran *meso* substituent would result in even higher activity. It would also be interesting to study how the replacement of the *para*-iodo or thiofuran groups at the *meso* position with other para-halide or furan groups would affect the activity.

From the 16 rosamine structures studied here, the most active compounds, 10 and 11, were at least 10-fold more potent than Rh123, a structurally similar compound whose anticancer properties have been extensively investigated [6]. Furthermore, our study also showed that compounds 10 and 11 showed greater cytotoxicity towards oral and nasopharyngeal cancer cells compared with immortalized normal cells of the same organ type. However, as other lipophilic cations have failed in clinical assessments, the true potential of compounds 10 and 11, or any other structurally similar analogs may only be ascertained in whole-animal preclinical studies.

Fluorescence microscopy studies showed that compound 11 localizes exclusively within the mitochondria. This, together with data from cell cycle analysis and the onset of apoptosis studies, suggests that compound 11 induced cell death through mitochondria-dependent apoptosis rather than through damaging the nucleic materials. The intracellular localization data here also agree with literature reports where the high mitochondrial transmembrane potential noted in cancer cells has resulted in the accumulation of lipophilic cations, such as the rosamine derivatives studied here, in mitochondria [7]. Overall, our results suggest that these compounds may offer a unique potential for the design of mitochondria-targeting agents that either directly kill or deliver cytotoxic drugs to selectively kill cancer cells.

Acknowledgements

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