Invest New Drugs
DOI 10.1007/s10637-010-9521-4

PRECLINICAL STUDIES

Salarin C, a member of the salarin superfamily of marine compounds, is a potent inducer of apoptosis

Nathalie Ben-Califa · Ashgan Bishara · Yoel Kashman · Drorit Neumann

Received: 24 June 2010 / Accepted: 11 August 2010

Summary The continuous emergence of new diseases and the development of drug-resistant cancers necessitate the development of new drugs with novel mechanisms of action. The richest marine source of natural anti-cancer products has been soft-bodied organisms that lack physical defenses against their predators, and hence rely on chemical defense mechanisms using cytotoxic secondary metabolites. Bio-guided (brine shrimp test) separation of $CHCl_3$-CH_3OH (1:1) extract from the Madagascar *Fascaplysinopsis* sp. sponge provided several new compounds. Here we focused on the biological activity of a panel of novel natural compounds, salarins A-J. Of these, salarin C was the most potent inhibitor of proliferation, as demonstrated on the K562 leukemia cell line. Salarin C-treated K562 cells underwent apoptotic death as monitored by cell-cycle analysis, annexin V/propidium iodide staining, cleavage of poly-ADP-ribose polymerase (PARP) and caspase 3, and caspase 9 levels. The experimental approach described herein is an essential step towards identifying the cellular pathway(s) affected by salarin C and producing potent synthetic derivatives of salarin C with potential future uses as basic research tools and/or drugs and drug leads.

Keywords Salarin C · Apoptosis · K562 cells · Caspase · Cell cycle

N. Ben-Califa · D. Neumann (✉)
Department of Cell and Developmental Biology,
Sackler Faculty of Medicine, Tel Aviv University,
Ramat Aviv 69978, Israel
e-mail: histo6@post.tau.ac.il

A. Bishara · Y. Kashman
School of Chemistry, Tel Aviv University,
Ramat Aviv 69978, Israel

Introduction

The continuous emergence of new diseases and the development of drug-resistant cancers are driving the development of innovative drugs with novel mechanisms of action. The richest marine source of natural anti-cancer products has been soft-bodied organisms that lack physical defenses against their predators, and therefore rely on chemical defense mechanisms involving cytotoxic secondary metabolites [1]. Despite the fact that most active natural and synthetic compounds have failed to be adopted as drugs because of toxicity and unfavorable activities, many of these compounds have been established as valuable drug leads or important research tools (reviewed in [1, 2]).

Among the mechanisms governing drug functions are inhibition of cell proliferation and/or induction of cell death. Cell death by apoptosis normally occurs as part of the organism's natural regulatory process, serving to establish and maintain proper control of cell turnover. Failure of cells to undergo apoptosis and/or their upregulated proliferation leads to various pathological states, including malignancies and autoimmune diseases (reviewed in [3]).

We previously investigated the Madagascar *Fascaplysinopsis* sp. sponge collected in Salary Bay, ~100 km north of Tulear. Bio-guided (brine shrimp test) separation of $CHCl_3$-CH_3OH (1:1) extract provided four groups of novel compounds—salarins, tulearins, taumycins and tausalarins—containing unique functionalized nitrogenous macrocycles [4–7]. In the present study, we examined the ability of a panel of natural salarins to inhibit proliferation and induce cell death in the K562 human leukemia cell line. We demonstrate that of the 10 salarins tested, salarin C is the most potent derivative in terms of its capacity to inhibit cell proliferation and induce cell death. Apoptosis

Published online: 24 August 2010

is proposed as a plausible mechanism, as depicted by cell morphology, cell-cycle analysis, annexin V/propidium iodide (PI) staining, caspase 3 and 9 cleavage, and cleavage of poly-ADP-ribose polymerase (PARP).

Materials and methods

Salarin congeners

Bio- (brine shrimp assay) and proton NMR-guided fractionation of the $CHCl_3/CH_3OH$ (1:1) extract of sponge using Sephadex LH-20 and silica gel chromatographies produced 10 salarins (Fig. 1). Each compound was characterized by MS, and 1D and 2D NMR spectra [5, 6, 8]. We used several batches of salarin C isolates, all of which displayed similar activities at the applied concentrations.

Antibodies

The following antibodies were used: anti-cleaved caspase 3 (Asp175, Cell Signaling), anti-poly ADP-ribose polymerase (PARP) (p09874, Serotec), and anti-actin (MAB1501, Chemicon International). Anti-caspase 9 (sc-7885) and anti-p21 (SC 397) were both obtained from Santa-Cruz Biotechnology.

Cell culture

The human chronic myeloid leukemia cell line K562 [9] was cultured in RPMI 1640 medium, supplemented with 10% (*v*/*v*) fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 μg/ml penicillin, at 37°C in a humidified 5% CO_2 incubator.

Colorimetric MTT assay

Cells (3×10^3) were seeded in triplicate in 96-well, flat-bottom culture plates and grown in the presence of the various compounds at different concentrations for 24, 48 and/or 72 h. Untreated cells served as controls. Cell viability was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) colorimetric assay. Aliquots of 10 μl MTT (5 mg/ml stock solution) were added to each well containing 100 μl of medium. After 4 h incubation, any formazan crystals formed were dissolved in acid/alcohol (140 μl 0.04 N HCl in isopropanol). The optical density (OD) was measured at 570 nm. Cell viability (%) was calculated as the ratio between the OD in the presence of compound and the OD in the absence of compound.

Flow cytometry analysis

Cellular DNA content was measured by flow cytometry. Cells cultured as described above, were treated for 24, 48, or 72 h with various concentrations of salarin C. The cells were then harvested, washed twice with ice-cold PBS, fixed in 70% ethanol and incubated with RNase A (100 μg/ml) at 37°C for 30 min. Propidium iodide (PI, 50 μg/ml in 0.1% Triton X-100 and 0.1% sodium citrate) was added to the samples 30 min before analysis in a

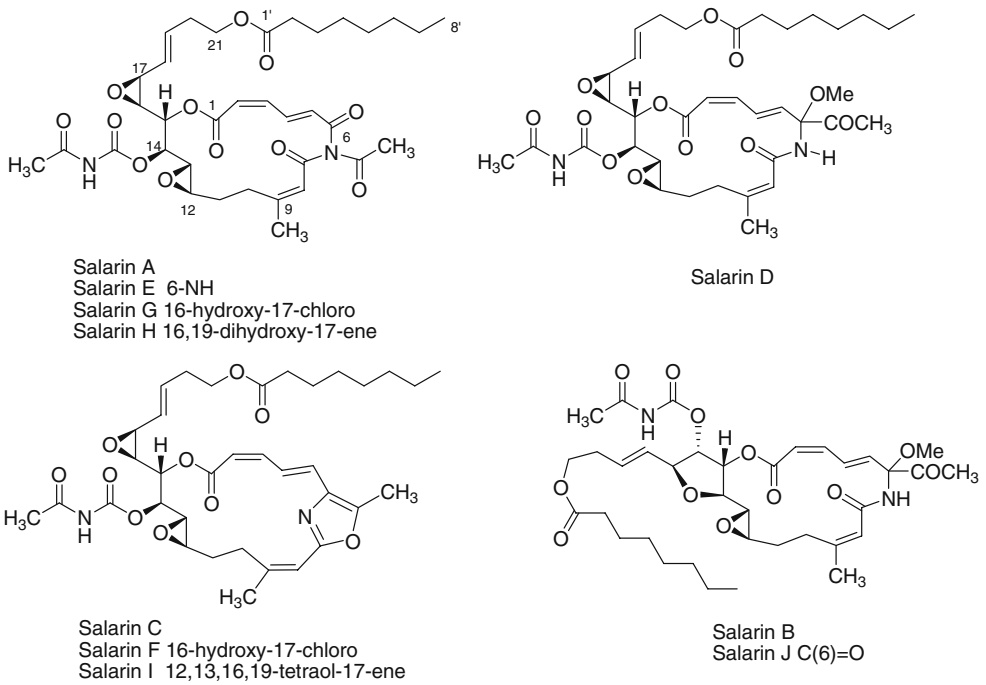


Fig. 1 Salarins A-J, four novel groups of nitrogenous marine macrolides

FACSort Flow Cytometer (BD). At least 1×10^4 cells were collected per sample and histograms were analyzed by WinMDI 2.8 software.

Annexin V binding was performed using the Mebcyto apoptosis kit as described by the manufacturer (MBL International, Japan). Following incubation for 15 min with anti-annexin V antibodies and PI, the cells were analyzed by flow cytometry. At least 1×10^4 events were recorded and represented as dot plots.

Western blot analysis

K562 cells were incubated with the salarins for the indicated periods of time. Cells were harvested and solubilized in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% (*v*/*v*) Triton X-100, 5 mM EDTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate and a protease inhibitor cocktail (Roche, Mannheim, Germany). Cell lysates were resolved by 12% SDS-polyacrylamide gel electrophoresis. Immunoblots were performed as previously described [10]. Briefly, nitrocellulose membrane filters (Schleicher and Shuell, Dassel, Germany) containing the transferred proteins were blocked with 5% non-fat milk in Tris-buffered saline (pH 7.4) containing 0.05% (*v*/*v*) Tween (TSBT) for 1 h. Membranes were then rinsed three times in TBST and incubated with the indicated primary and secondary antibodies, followed by ECL signal detection on the Western blots. Actin levels were assessed as a loading control.

Results

Effect of salarins on viability of K562 cells

K562 is a chronic myeloid leukemia cell line with rapid growth rate and poor differentiation [9]. Initially, we tested a panel of natural salarins (Fig. 1) for their effect on proliferation of K562 cells. K562 cells (3×10^3 cells) were incubated with salarins A-J (at 1 μM) for 3 days and cell viability was assessed by the MTT method. As seen in Fig. 2, salarin C was the most potent inhibitor of cell proliferation and inducer of cell death: after 3 days in the presence of salarin C, no viable cells could be detected in the culture. After 3 days in culture, salarins B, D, E, H and J inhibited 30 to 50% proliferation of the K562 cells whereas, salarins A, F, G and I displayed minimal (<20% inhibition) or no effect on the cells. These results thus directed our focus to salarin C with respect to its effect on cell viability and proliferation. To determine time and dose response of salarin C on cell viability, K562 cells (3×10^3) were incubated in the presence of 0.001, 0.005, 0.01, 0.05, 0.1 or 1 μM salarin C for 24, 48 or 72 h. As shown in Fig. 3, incubation of the

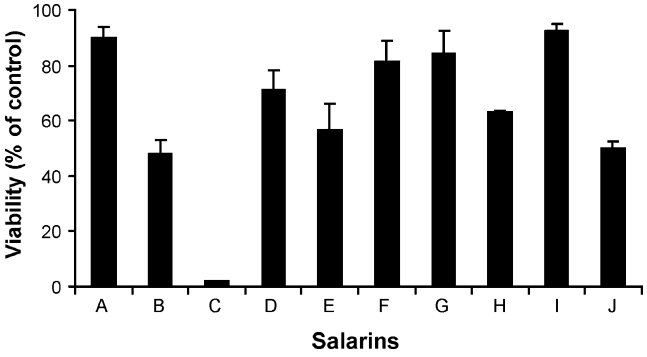


Fig. 2 Effect of salarins on K562 cell viability. K562 cells (3×10^3 in 100 μl) were treated with 1 μM of each salarin derivative for 72 h. Cell viability was determined by using the MTT assay and expressed as percent viability of control, non-treated cells. Results represent the mean ± SD of three independent experiments

cells for 24 h in the presence of 0.1 μM salarin C resulted in 50% viability. Salarin C at 0.001 μM had no measurable effect on cell viability during the 72 h span of the experiment. Note that salarin C has been shown to potently kill several tumor cell lines, as revealed by an in vitro screen of 60 cell lines [11] performed by the National Cancer Institute (data not shown).

Salarin C induces apoptosis of K562 cells

To characterize salarin C-mediated cell death, K562 cells were incubated in the presence of 0.001, 0.005, 0.01, 0.05, 0.1 or 1 μM salarin C, or in the absence of compound (control), for 24, 48 or 72 h. Cells were then stained with PI and subjected to flow cytometry analysis. As shown in Fig. 4, treatment of the cells with salarin C at 0.1 μM and 0.05 μM resulted in 30 and 26% apoptosis, respectively, even after 24 h (Fig. 4b, SubG1). By 72 h, most of the cells were dead in the presence of both concentrations. At lower salarin C concentrations (0.001–0.01 μM), apoptotic cells

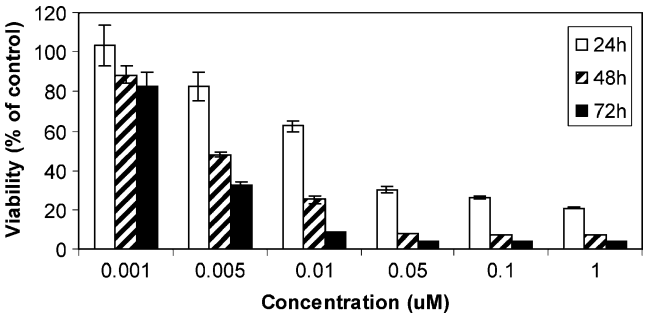
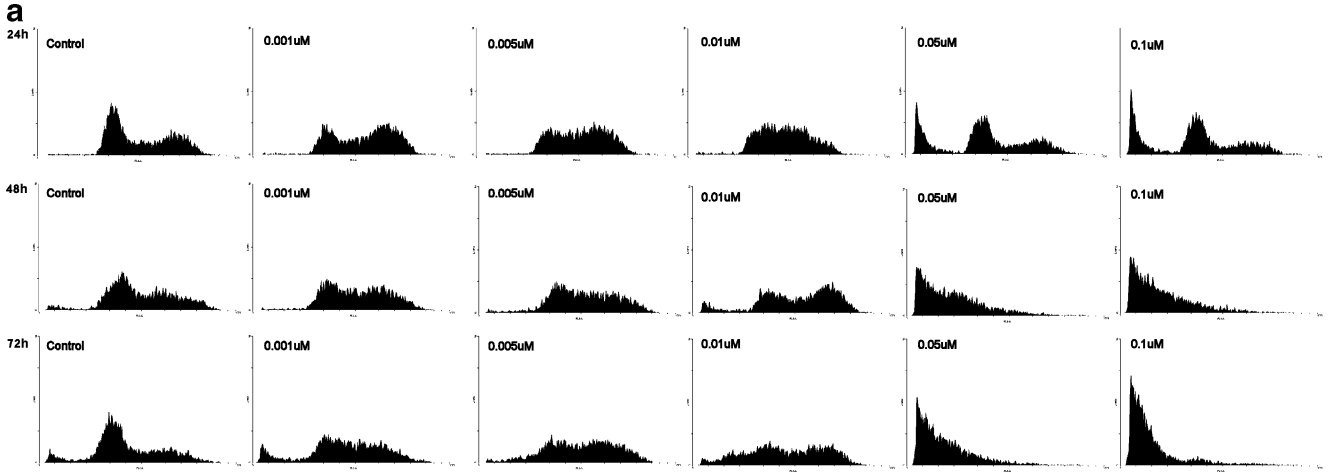


Fig. 3 Salarin C reduces viability of K562 cells in a dose-dependent manner. K562 cells (3×10^3 in 100 μl) were treated with the indicated concentrations of salarin C for 24, 48, and 72 h. Cell viability was determined by MTT assay. Viability of salarin C-treated cells is expressed as percent of viability of the non-treated (control) cells. Results represent the mean ± SD of three independent experiments

a



b

Salarin C	Control			0.001uM			0.005uM			0.01uM			0.05uM			0.1uM		
Time	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h
Sub G1 (%)	1.4	5.8	9.2	1.5	3.9	12.9	2.0	5.3	7.6	3.2	9.8	17.7	26.0	81.8	65.4	30.1	87.4	71.7
G0/G1 (%)	45.3	45.3	52.8	28.0	40.7	33.7	31.3	38.7	30.3	38.2	31.8	30.8	38.3	14.0	24.3	38.5	8.4	20.4
S (%)	21.9	27.7	26.4	28.7	33.5	40.1	40.5	31.9	41.8	42.8	28.6	33.7	16.4	3.6	8.7	15.9	2.7	7.0
G2/M (%)	31.4	21.4	11.8	41.8	22.1	13.6	26.2	20.5	20.5	29.8	29.8	17.8	19.3	0.8	1.5	15.6	1.5	1.0

Fig. 4 Salarin C effects on K562 cell cycle. K562 cells (3×10^5 in 10 ml) were cultured in the absence (control) or presence of the indicated concentrations of salarin C. Following incubation, cells were harvested, permeabilized, stained with PI and analyzed by flow cytometry after 24, 48 and 72 h (**a**). Cell-cycle distribution in % for the above representative experiment (of $n=3$) is indicated (**b**)

were only discerned after 48 h of treatment. The effect of salarin C on the cell cycle was dose- and time-dependent. Notably, G2/M accumulation was observed at 0.001 μM salarin C after 24 h, whereas treatment with 0.01 μM salarin C led to G2/M accumulation after 48 h. We thus propose that following treatment with salarin C, cells from G2/M are directed to apoptosis.

Induction of apoptosis by salarin C was also analyzed by annexin V/PI staining. Cells were cultured overnight in the presence or absence of 0.2 μM, 0.1 μM or 0.01 μM salarin C and then stained with annexin V and PI, and analyzed by flow cytometry (Fig. 5). Whereas the percentage of apoptotic cells in the control culture was 2.4, the percentage of apoptotic cells (annexin V positive/PI negative) was 63.2, 53.2 and 8.6 for 0.2 μM, 0.1 μM and 0.01 μM salarin C-treated cells, respectively (Fig. 5).

Salarin C-mediated apoptosis was also monitored by Western blot analysis of cell lysates for caspase 9, cleaved PARP and cleaved caspase 3 (Fig. 6). Cells were treated for 24 or 48 h in the presence or absence of 0.1 μM, 0.05 μM, 0.01 μM or 0.005 μM salarin C. As depicted in Fig. 6, after 24 h of salarin C treatment, PARP and caspase 3 cleavage was evident at salarin C concentrations of 0.01 μM and higher. A decrease in caspase 9 levels was evident in cells subjected to 0.1 μM and 0.05 μM salarin C. No detectable change in the levels of these markers was evident at 0.005 μM of the compound. Notably, after 48 h of incubation in the presence of salarin C, cleavage of both PARP and caspase 3 was evident at all concentrations (up to 0.005 μM) of the compound. The decrease in caspase 9 was evident up to 0.01 μM salarin C. The lower sensitivity of caspase 9 immunoblot analyses at both 24 and 48 h may have resulted from the fact that its antibody detects reductions in the pool of caspase 9, whereas cleaved PARP and caspase 3 reflect the *de novo* generation of the corresponding proteolytic fragments.

The cyclin-dependent kinase inhibitor p21 (also known as p21WAF1/Cip1) promotes cell-cycle arrest in response to activation of p53 (reviewed in [12]) and/or other stimuli (reviewed in [13]). The salarins' ability to inhibit proliferation thus raised the question of p21's involvement. Western blots of lysates from K562 cells treated with the compounds (1 μM salarins A, B or E-J for 3 days or 0.1 μM salarin C for 16 h) were probed for the expression of p21. As shown in Fig. 7, cells treated with salarins B, E and J had robust levels of p21, whereas p21 levels in cells treated with salarins D and H were considerably lower. In cells that were treated with salarins A, C, F, G and I, p21 could not be detected. Finally, cleaved caspase 3 was only detected in salarin C-treated cells (Fig. 7).

Discussion

We compared a panel of natural salarins (A-J) for their effect on the proliferation and viability of K562 cells, and show that among the salarins tested, salarin C was the most effective. It

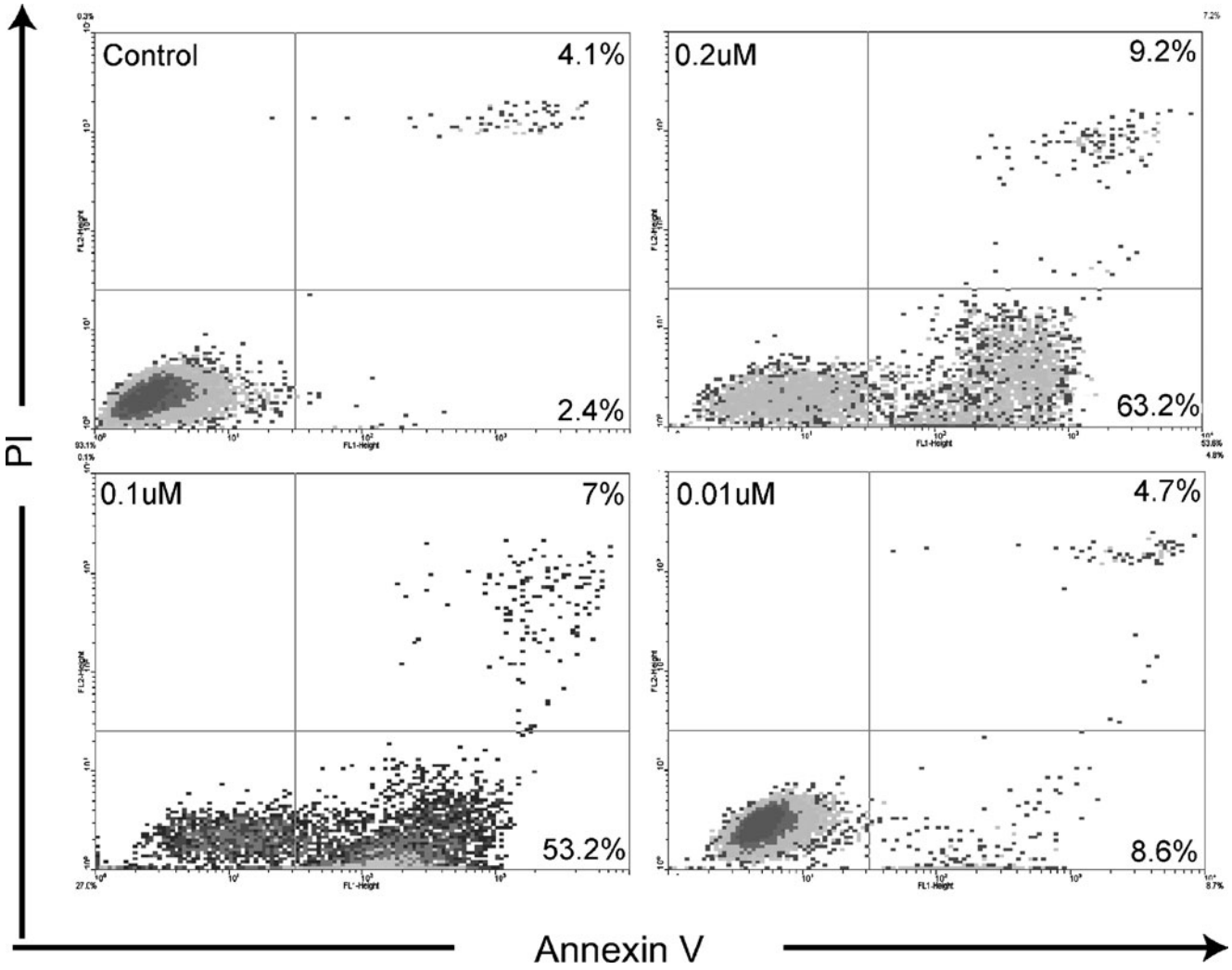


Fig. 5 Salarin C induces apoptosis of K562 cells. K562 cells (1×10^6 in 10 ml) were incubated for 16 h in the absence (control) or presence of salarin C (0.2 μM, 0.1 μM, or 0.01 μM). Following incubation, cells were harvested, stained with annexin V-FITC and PI and analyzed by flow cytometry. The proportion of early apoptotic cells (annexin V positive/PI negative) and dead cells (annexin V positive/PI positive) are indicated in the lower and upper right quadrants of each graph, respectively. Depicted results are representative of three independent experiments displaying similar results

reduced cell viability and was a potent inducer of apoptotic cell death. This was demonstrated by a panel of biochemical assays including MTT, cell-cycle analysis, annexin V/PI staining, cleavage of caspase 3 and PARP and caspase 9 levels.

The availability of a panel of salarins differing in particular chemical moieties enabled us to relate biological activity to selected structural features. Salarin C, the most potent among the 10 salarins, possesses a variety of unique functional groups. These include a 2,4-doubly conjugated oxazole ring, a *2Z,4E*-2,3,4,5-unsaturated lactone, a vinyl epoxide and another non-conjugated epoxide, as well as an acetyl carbamate. Among these functional moieties, the most sensitive is the conjugated oxazole ring that changes to a triacylamine group within 24 h in the air under light, producing salarin A. Second in reactivity is the vinyl epoxide which produces a hydroxy allyl cation, initiating a complex chain of reactions. Salarins B and J, which were also biologically active, possess a unique α to the *N*-atom doubly substituted unsaturated lactam (Fig. 1). These salarins also differ from salarin C in the second half of the macrolide. Salarins E and H were far less potent than salarins C, B and J. They possess the naturally unprecedented di- and triacylamine moieties, respectively, while the rest of the molecules are identical to their counterparts in salarins C and A. Notably, salarin A was less active than salarins E and H.

It thus appears that the high reactivity of salarin C requires a subtle and specific arrangement of the various functional groups of this molecule. Targeted chemical modifications are currently being performed to pinpoint functional groups in salarin C that are essential for its biological activity. In addition, synthetic derivatization of salarin C is underway to shed light on the structure-activity

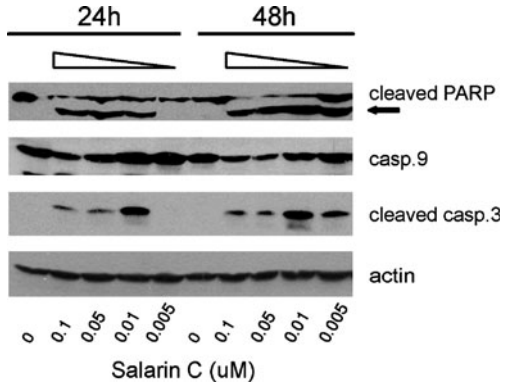


Fig. 6 Salarin C induces caspase 3, caspase 9 and PARP cleavage. K562 cells (1×10^6 cells in 10 ml) were incubated for 24 and 48 h in the absence (0) or presence of 0.1, 0.05, 0.01 and 0.005 μM salarin C. Following incubation, cells were harvested and equal amounts of total cell lysates were subjected to 12% SDS-PAGE followed by immunoblot analyses with anti-PARP, anti-caspase 9 and anti-cleaved caspase 3 antibodies. Actin was employed as a loading control. Data are representative of three independent experiments displaying similar results

relationship. This analysis will eventually facilitate the generation of active synthetic moieties.

The effects of salarin C on cell viability and proliferation were dose- and time-dependent. At high salarin C concentrations (0.1–0.05 μM), cell apoptosis was evident; at low concentrations (0.001–0.01 μM), inhibition of proliferation (G2/M accumulation) could be discerned. We thus propose that the G2/M cells are being directed to apoptosis.

While salarin C induced the cleavage of caspase 3 and apoptosis of the cells, the other salarins (B, D, E, H, and J) reduced cell viability, but did not lead to caspase 3 cleavage. Notably, salarins B, D, and J, which all conferred significant upregulation of p21, led to cell-cycle arrest in G_1 (data not shown). The G2/M arrest conferred by salarin C, coupled to the inability to detect salarin C-associated elevation in p21, discerns the latter's mode of action from

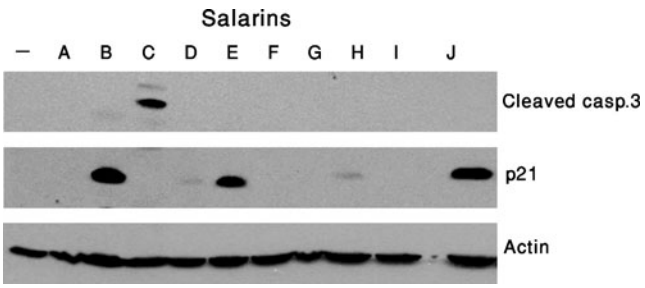


Fig. 7 Effects of salarins on p21 expression. K562 cells (3×10^5 in 10 ml) were incubated for 3 days in the absence (-) or presence of 1 μM salarins A, B, D-J. Salarin C (0.1 μM) was added to 1×10^6 cells for 16 h. Following the incubation, cells were harvested, and equal amounts of total cell lysates were resolved on 12% SDS-PAGE followed by immunoblot analyses with anti-p21 and anti-cleaved caspase 3 antibodies. Actin levels were employed as a loading control. Data depict a representative experiment of three independent experiments displaying similar results

that of the other salarins. Lack of p21 in the salarin C-treated cultures may also indicate that p21 is degraded by activated caspase 3 [14]. As K562 cells do not express p53 [15], elevation of p21, as well as the salarin C-induced apoptosis, are most likely not mediated by p53. The precise cellular pathway(s) [16, 17] by which salarin C leads to apoptotic cell death has not yet been deciphered, and is the focus of our ongoing research.

Salarin C's effect on the cells was rapid and irreversible. Salarin C-mediated apoptosis (annexin V/PI staining) was detected even when the compound was removed after 4 h of incubation and the cells were left in culture for an additional 16 h (data not shown). The rapid effects of salarin C were also observed in HeLa cells in which caspase 3 cleavage was detected as early as 4 h after exposure of the cells to the compound (data not shown). In both HeLa and K562 cells after 4 h of salarin C treatment, we detected release of cytochrome C from the mitochondria, indicating mitochondrial damage (data not shown). Whether mitochondria are the primary target of salarin C, or a secondary one, remains to be resolved.

Although most active natural and synthetic compounds fail to be adopted as drugs because of toxicity and unfavorable activities, many are useful as drug leads, or become important cell biology research tools. An ideal approach to anti-cancer therapy is the use of "tailor-designed" drugs for each particular tumor. Efficient generation of such drugs, tailored to individual cancers, necessitates identification of their intracellular targets and the signaling pathways that they impair. Such knowledge would enable synthetic manufacture of these drugs, as well as molecular manipulations, as needed. The finding that salarin C inhibits cell proliferation and induces apoptosis in a dose- and time-dependent manner renders it an attractive research tool for basic science, as well as a potential candidate for clinical applications, the extent of which remains to be determined. The experimental approach described herein presents an essential step towards identification of the cellular pathway(s) affected by salarin C and production of potent synthetic derivatives of salarin C with future applications as basic research tools and/or drugs and drug leads.

Acknowledgements The authors thank Prof. Ina Fabian for critically reviewing the manuscript. The research was supported by the Israeli Science Foundation (ISF), Grant 365/09 to YK.

Conflict of interest statement None declared.

References

1. Napolitano JG, Daranas AH, Norte M, Fernandez JJ (2009) Marine macrolides, a promising source of antitumor compounds. Anticancer Agents Med Chem 9(2):122–137

2. Nobili S, Lippi D, Witort E, Donnini M, Bausi L, Mini E, Capaccioli S (2009) Natural compounds for cancer treatment and prevention. Pharmacol Res 59(6):365–378
3. Merino D, Bouillet P (2009) The Bcl-2 family in autoimmune and degenerative disorders. Apoptosis 14(4):570–583
4. Bishara A, Rudi A, Aknin M, Neumann D, Ben-Califa N, Kashman Y (2008) Taumycins A and B, two bioactive lipodepsipeptides from the madagascar sponge fascaplysinopsis sp. Org Lett 10(19):4307–4309
5. Bishara A, Rudi A, Aknin M, Neumann D, Ben-Califa N, Kashman Y (2008) Salarins A and B and tulearin A: new cytotoxic sponge-derived macrolides. Org Lett 10(2):153–156
6. Bishara A, Rudi A, Aknin M, Neumann D, Ben-Califa N, Kashman Y (2008) Salarin C, a new cytotoxic sponge-derived nitrogenous macrolide. Tetrahedron Lett 49:4355–4358
7. Bishara A, Rudi A, Goldberg I, Aknin M, Neumann D, Ben-Califa N, Kashman Y (2009) Tausalarin C: a new bioactive marine sponge-derived nitrogenous bismacrolide. Org Lett 11(16):3538–3541
8. Bishara A, Rudi A, Aknin M, Neumann D, Ben-Califa N, Kashman Y (2010) Salarins D-J, seven new nitrogenous macrolides from the madagascar sponge fascaplysinopsis sp. Tetrahedron 66(24):4339–4345
9. Lozzio BB, Lozzio CB (1977) Properties of the K562 cell line derived from a patient with chronic myeloid leukemia. Int J Cancer 19(1):136
10. Cohen J, Altaratz H, Zick Y, Klingmuller U, Neumann D (1997) Phosphorylation of erythropoietin receptors in the endoplasmic reticulum by pervanadate-mediated inhibition of tyrosine phosphatases. Biochem J 327(2):391–397
11. Shoemaker RH (2006) The NCI60 human tumour cell line anticancer drug screen. Nat Rev Cancer 6(10):813–823
12. Kuribayashi K, El-Deiry WS (2008) Regulation of programmed cell death by the p53 pathway. Adv Exp Med Biol 615:201–221
13. Abbas T, Dutta A (2009) P21 in cancer: intricate networks and multiple activities. Nat Rev Cancer 9(6):400–414
14. Zhang Y, Fujita N, Tsuruo T (1999) Caspase-mediated cleavage of p21Waf1/Cip1 converts cancer cells from growth arrest to undergoing apoptosis. Oncogene 18(5):1131–1138
15. Lubbert M, Miller CW, Crawford L, Koeffler HP (1988) P53 in chronic myelogenous leukemia. Study of mechanisms of differential expression. J Exp Med 167(3):873–886
16. Caroppi P, Sinibaldi F, Fiorucci L, Santucci R (2009) Apoptosis and human diseases: mitochondrion damage and lethal role of released cytochrome C as proapoptotic protein. Curr Med Chem 16(31):4058–4065
17. Constantinou C, Papas KA, Constantinou AI (2009) Caspase-independent pathways of programmed cell death: the unraveling of new targets of cancer therapy? Curr Cancer Drug Targets 9 (6):717–728