

# Selective cytotoxic activity of the marine-derived batzelline compounds against pancreatic cancer cell lines

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Pancreatic cancer is the fourth leading cause of cancer death in the United States. The prognosis of the disease is very negative, because the cancer will be usually metastasized by the time a patient manifests symptoms. Although combination therapy shows some promise, new drugs to treat the disease are needed. Given our interest in finding new therapies for pancreatic cancer, we sought to determine whether the known cytotoxic activity of the batzellines extended to pancreatic cancer cell lines. The batzellines are pyrroloiminoquinones alkaloids obtained from the deep-water Caribbean sponge *Batzella* sp (family Esperipsidae, order Poecilosclerida). We show here that batzellines exhibit selective cytotoxicity towards the pancreatic cancer cell lines AsPC-1, Panc-1, BxPC-3, and MIA PaCa2 compared with the normal African green monkey kidney epithelial cell line Vero. The batzellines cause cytotoxicity by inducing cell cycle arrest that is mediated by their ability to intercalate into DNA and/or inhibit topoisomerase II activity. The cytotoxic abilities of

isobatzellines A and C against pancreatic cancer cell lines, their low toxicity against normal cells, and their reported ability to be synthesized makes them interesting compounds with potential chemotherapeutic effects that may merit further research. *Anti-Cancer Drugs* 20:149–155 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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## Introduction

The American Cancer Society estimates that 37 170 new cases of pancreatic cancer were detected and about 33 370 deaths because of pancreatic cancer occurred in 2007, making it the fourth leading cause of cancer death in the United States [1]. The grim prognosis of pancreatic cancer results from the fact that by the time a patient displays symptoms, the cancer has already metastasized [1]. Treatment of pancreatic cancer involves surgery, radiation therapy, chemotherapy, or a combination of the three. The current chemotherapy for advanced pancreatic cancer is gemcitabine, a drug that inhibits DNA synthesis [1–3]. Another lead compound in the treatment of pancreatic cancer is 5-fluorouracil. Combination therapy using gemcitabine and 5-fluorouracil is only slightly more effective than gemcitabine alone [2]. Furthermore, there is no good therapy to treat pancreatic tumors that become refractory to gemcitabine [3]. The prognosis of pancreatic cancer patients under current treatments is poor, and new drugs to treat the disease are needed.

The batzellines are pyrroloiminoquinones alkaloids obtained from the deep-water Caribbean sponge *Batzella* sp (family Esperipsidae, order Poecilosclerida) [4–6] that exhibit antifungal activity as well as cytotoxicity against murine leukemia cells [4]. They are structurally related to other marine compounds such as the makaluvamines and the

discorhabdins that exhibit strong cytotoxicity against many different cancer cell lines [7]. Furthermore, the makaluvamines exhibit potent antitumor activity and are currently under intense research as potential therapeutics. Makaluvamine A and C effectively diminished tumor load of nude mice injected with human ovarian cancer tumors [8] and makaluvamine H reduced tumor load of nude mice injected with nasopharyngeal cancer cells [9].

A comprehensive study of marine pyrroloiminoquinones in the NCI 60 cell line panel showed that the cytotoxic activity of these compounds extends to many human cancers [10]. However, of the batzellines used in this study, only isobatzelline C and isobatzelline E were included in that study and their mechanism of action was not ascertained. Furthermore, pancreatic cancer cell lines were not included in that panel [10]. Given our interest in finding new therapies for pancreatic cancer, we sought to determine whether the cytotoxic activity of the batzellines extended to pancreatic cancer cell lines, and to determine the mechanism by which this cytotoxicity was induced.

## Materials and methods

### Reagents

Batzelline A, batzelline B, isobatzelline A, isobatzelline C, isobatzelline D, isobatzelline E, secobatzelline A, and

secobatzelline B are part of the Harbor Branch Oceanographic Institute library of compounds isolated from marine organisms. Methanol and isopropanol were purchased from Fisher Scientific, Fair Lawn, New Jersey, USA. Tris, sodium chloride, 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide, bromophenol blue, and 5-fluorouracil were purchased from Sigma Chemical Co., St. Louis, Missouri, USA.

#### Cell culture

The pancreatic carcinoma cell lines AsPC-1 (CRL-1682), Panc-1 (CRL-1469), BxPC-3 (CRL-1687), and MIA PaCa2 (CRL-1420), as well as the normal African green monkey kidney epithelial cell line Vero (CRL-1587) were obtained from American Type Culture Collection (Manassas, Virginia, USA) grown, aliquoted, and stored under liquid nitrogen. Aliquots of those cells were thawed and grown in RPMI-1640 with 10% fetal bovine serum, 0.11 mg/ml sodium pyruvate, 4.5 g/l D-glucose, 18 mmol/l HEPES buffer, 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, 0.25 µg/ml amphotericin B, 2 mmol/l L-glutamine, and 50 µg/ml gentamicin (complete RPMI). Cells were maintained in a humidified incubator at 37°C and 5% CO<sub>2</sub>.

#### Cell viability assay (methylthiazoletetrazolium)

Six thousand cells/well in 200 µl of media were plated in a 96-well tissue culture plate. Cells were allowed to adhere for 24 h. At the end of this incubation, 100 µl of medium was removed from each test well and 100 µl of medium containing treatment was added. Treatment consisted of eight 1:2 serial dilutions ranging from 5 to 0.0391 µg/ml of the batzelline compounds, 5-fluorouracil, or media with methanol. The cells were then incubated for 72 h at 37°C and 5% CO<sub>2</sub>. After this incubation, 75 µl of a 5 mg/ml solution of methylthiazoletetrazolium was added to each well. The cells were then incubated for 3 h at 37°C. At the end of this incubation, cells were centrifuged, the supernatant was removed, and 200 µl of acidified isopropyl alcohol (1:500 solution of hydrochloric acid to isopropanol) was added to each well. The plates were then shaken for 15 min and the absorbencies of these solutions measured at 570 nm with a plate reader (NOVOstar, BMG Labtech Inc., Durham, North Carolina, USA). The data were normalized to the vehicle control and the normalized data was subjected to nonlinear regression assuming a variable slope to calculate the dose needed to produce 50% cytotoxicity (IC<sub>50</sub>). Samples were tested in duplicate per assay and the assay was performed in quadruplicate.

#### Cell cycle analysis

AsPC-1 cells were trypsinized and split onto a 6-well plate where they were allowed to adhere for 24 h. Cells were then treated for 48 h with 5 or 25 µg/ml of the batzellines, the vehicle control, no treatment, or 100 nmol/l paclitaxel as a positive control. At the end of

treatment, cells were trypsinized, pelleted, fixed with ice-cold ethanol, and incubated at -20°C for an hour. Cells were then washed and stained with propidium iodide in the presence of RNase A for 30 min at 37°C. Cells were then transferred to tubes and analyzed in a flow cytometer. Samples were tested once per experiment and the experiment was performed in triplicate.

#### Decatenation reaction

The decatenation reaction was carried out using a kit purchased from TopoGEN Inc., Port Orange, Florida, USA (Catalog #1001-2) and following the manufacturer's instructions. All reactions, with the exception of a positive control lacking topoisomerase II, contained 0.2 µg kinetoplast DNA, 1U topoisomerase II, and buffer (4.5 mmol/l ATP, 0.45 M Tris-HCl, 1.1 M NaCl, 91 mmol/l MgCl<sub>2</sub>, and 4.5 mmol/l dithiothreitol). In addition, some reactions contained either 5 or 25 µg/ml of each of the batzelline compounds. Other reactions contained 100 µmol/l etoposide (TopoGEN, Pt Orange, Florida, USA), as a positive control. The vehicle control reaction for the batzellines contained 0.1 or 0.5 µl of methanol (Fisher Scientific, Fair Lawn, New Jersey, USA), whereas the one for the etoposide contained 1 µl dimethylsulfoxide (American Type Culture Collection, Rockville, Maryland, USA). All reactions were brought to a final volume of 20 µl with deionized water. The samples were then incubated at 37°C for 30 min in a water bath. The reactions were terminated with the addition of 4 µl 5X stop buffer/loading dye (5% Sarkosyl, 0.125% bromophenol blue, 25% glycerol), and loaded into 1% agarose gel containing 0.5 µg/ml ethidium bromide. The samples were resolved through electrophoresis and viewed with an ultraviolet light box (Stratagene, La Jolla, California, USA). An image of the gel was obtained by using Eagle Sight software (Stratagene) and analyzed using Image J software (NIH, Bethesda, Maryland, USA). Optical density measurements were plotted using Microsoft Excel. Samples were tested once per experiment and the experiment was performed seven times.

#### DNA intercalation assay

This assay was performed as described by Dijoux *et al.* [10]. Briefly, 0.5 µg of genomic salmon testes DNA (Calbiochem, La Jolla, California, USA) along with 1.27 µmol/l ethidium bromide were plated on a black 96-well plate and allowed to equilibrate for half an hour at room temperature. Then, 5 or 25 µg/ml of the batzellines, the vehicle control methanol, 100 µmol/l etoposide (negative control), or 1 µmol/l doxorubicin (positive control) were added and allowed to equilibrate for half an hour at room temperature. Fluorescence was read at an excitation of 340 and an emission at 590 with a plate reader (NOVOstar, BMG Labtech Inc.). Samples were tested once per experiment and the experiment was performed in triplicate.

## Statistics

Statistical analysis of the data sets to determine mean, standard deviation, variance, and standard error of the mean was performed by using Microsoft Excel. To determine whether inhibition of target expression by our compounds was statistically different from the untreated samples and/or the vehicle control, these two sets of data were compared using the Student's *t*-test. A *P* value  $\leq 0.05$  was considered significant. Outliers were detected through the Grubbs test.

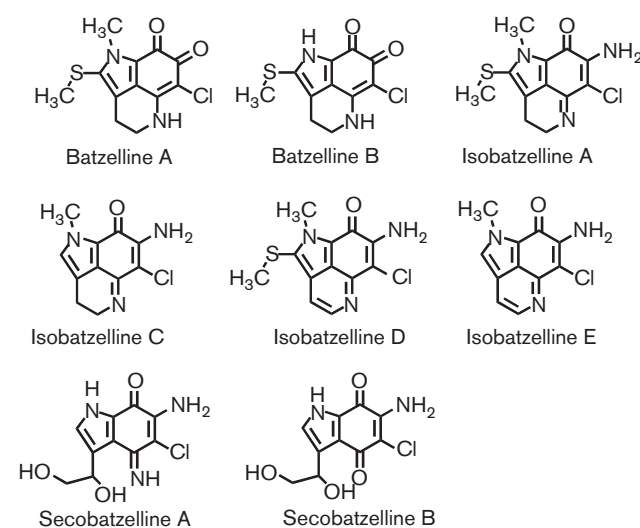
## Results

The isolation and chemical characterization of the batzellines used in this study were the subject of earlier publications [4–6]. They all share planar structures that contain a variant of a tricyclic pyrrolo[4,3,2-de]quinoline core ring structure (Fig. 1). The presence or absence of

distinct side chains is what differentiates members of this family most.

The cytotoxic effects of the batzellines were determined in four different pancreatic cell lines – Panc-1, AsPC-1, BxPC-3, and MIA-PaCa2 – as well as in the Vero cell line, an epithelial cell line from the kidney tissue of an African green monkey. These pancreatic cancer lines are thought to represent the spectrum of pancreatic cancer's metastatic potential. The results (Table 1) show that isobatzelline A, isobatzelline C, isobatzelline D, and secobatzelline A exhibited cytotoxicity in all pancreatic cell lines with a minimum concentration of compound required to produce IC<sub>50</sub>) of less than 10  $\mu\text{mol/l}$ . Furthermore, their cytotoxic effects on AsPC-1, BxPC-3, and MIA PaCa2 were far superior to 5-fluorouracil, a current treatment for pancreatic cancer. Interestingly, the batzellines exhibited much less cytotoxicity in the Vero cells, suggesting a preferential cytotoxic ability towards tumor cell lines.

Fig. 1



Structures of the batzellines.

Next, we sought to determine the mechanism by which the batzellines were inducing cell death. Two arbitrary doses, 5 and 25  $\mu\text{g/ml}$ , were chosen for the mechanism of action studies. These doses are higher than the IC<sub>50</sub> representing approximately 20 and 100  $\mu\text{mol/l}$ , respectively, of the batzellines (see Table 2 for exact conversions), but allowed us to recapitulate cell death in a shorter time. As reports of structurally related marine pyrroloiminoquinones cite inhibition of topoisomerase II as their mechanism of action [8–10], we decided to test whether the batzellines shared this property. Topoisomerase II is an enzyme that allows DNA strands to move through each other for synthesis of the lagging strand during DNA replication. Inhibiting this enzyme prevents the synthesis of the lagging strand thus stopping DNA replication and inducing cell cycle arrest and apoptosis [11]. Topoisomerase II inhibitors are widely used as chemotherapy in the treatment of cancer [12]. Through an in-vitro decatenation reaction of kinetoplast

Table 1 Cytotoxicity of batzellines in pancreatic cancer cell lines

Compound	MW	IC <sub>50</sub> ( $\mu\text{mol/l}$ )				
		Panc-1	AsPC-1	BxPC-3	MIA PaCa2	Vero
Batzelline A	282.75	>17.7	>17.7	>17.7	>17.7	>17.7
Batzelline B	268.75	>18.6	>18.6	>18.6	>18.6	>18.6
Isobatzelline A	281.75	9.4 $\pm$ 0.5	1.7 $\pm$ 0.4	2.4 $\pm$ 0.2	4.3 $\pm$ 0.2	>17.7
Isobatzelline C	235	10 $\pm$ 0.4	1.7 $\pm$ 0.2	1.3 $\pm$ 0.2	2.3 $\pm$ 0.1	>21.3
Isobatzelline D	279	5.7 $\pm$ 0.3	1.5 $\pm$ 0.2	1.5 $\pm$ 0.2	2.7 $\pm$ 0.3	15.7 $\pm$ 1.2
Isobatzelline E	233	>21.5	>21.5	>21.5	>21.5	>21.5
Secobatzelline A	255	10.4 $\pm$ 1.2	3.6 $\pm$ 0.8	4.1 $\pm$ 0.1	5.6 $\pm$ 0.7	14.0 $\pm$ 1.5
Secobatzelline B	256	17.4 $\pm$ 0.3	>19.5	>19.5	>19.5	>19.5
5-Fluorouracil	130.1	5.7 $\pm$ 0.7	>38.4	5.6 $\pm$ 1.6	8.3 $\pm$ 0.4	5.2 $\pm$ 0.3
Gemcitabine <sup>a</sup>	299.66	7.2	>250	>250	14.5	NA

*n* = 4  $\pm$  SE.

IC<sub>50</sub>, 50% cytotoxicity; MW, molecular weight; NA, not available.

<sup>a</sup>Values obtained from Li *et al.* [14].

DNA, we determined that batzelline A, batzelline B, isobatzelline A, isobatzelline E, and secobatzelline A significantly inhibited topoisomerase II activity at a dose of 25 µg/ml (Fig. 2). No significant topoisomerase II

**Table 2 Summary of activity of batzellines on AsPC-1 cells at 25 µg/ml**

Compound	µmol/l	% Inhibition topoisomerase II	Cell cycle arrest	% DNA intercalation
Batzelline A	88.42	58*	S phase	18
Batzelline B	93.02	63*	S phase	21
Isobatzelline A	88.73	36*	S phase	54**
Isobatzelline C	106.38	27	S phase	56**
Isobatzelline D	89.61	26	S phase	47**
Isobatzelline E	107.30	95*	G2	27
Secobatzelline A	98.04	61*	S phase	34**
Secobatzelline B	97.66	13	S phase	17

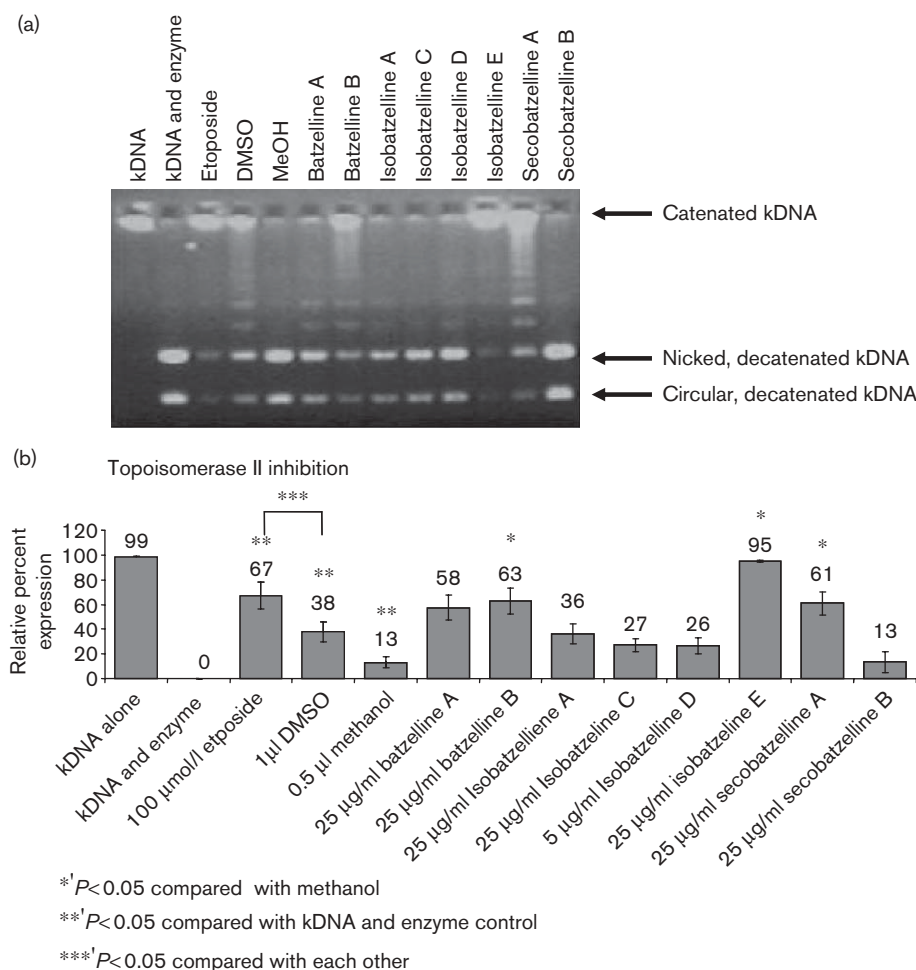
\* $P \leq 0.05$ .

\*\* $P \leq 0.005$ .

inhibition was found at the lower dose of 5 µg/ml (data not shown). The best inhibitors of topoisomerase II activity were isobatzelline E (95% inhibition) and batzelline B (63% inhibition), which are not among the most cytotoxic of the batzellines at 72 h of treatment. Isobatzelline A, C, and D which exhibit the highest cytotoxicity against all cell lines tested did not inhibit topoisomerase II under the conditions tested, suggesting an alternative mechanism of action for those batzellines.

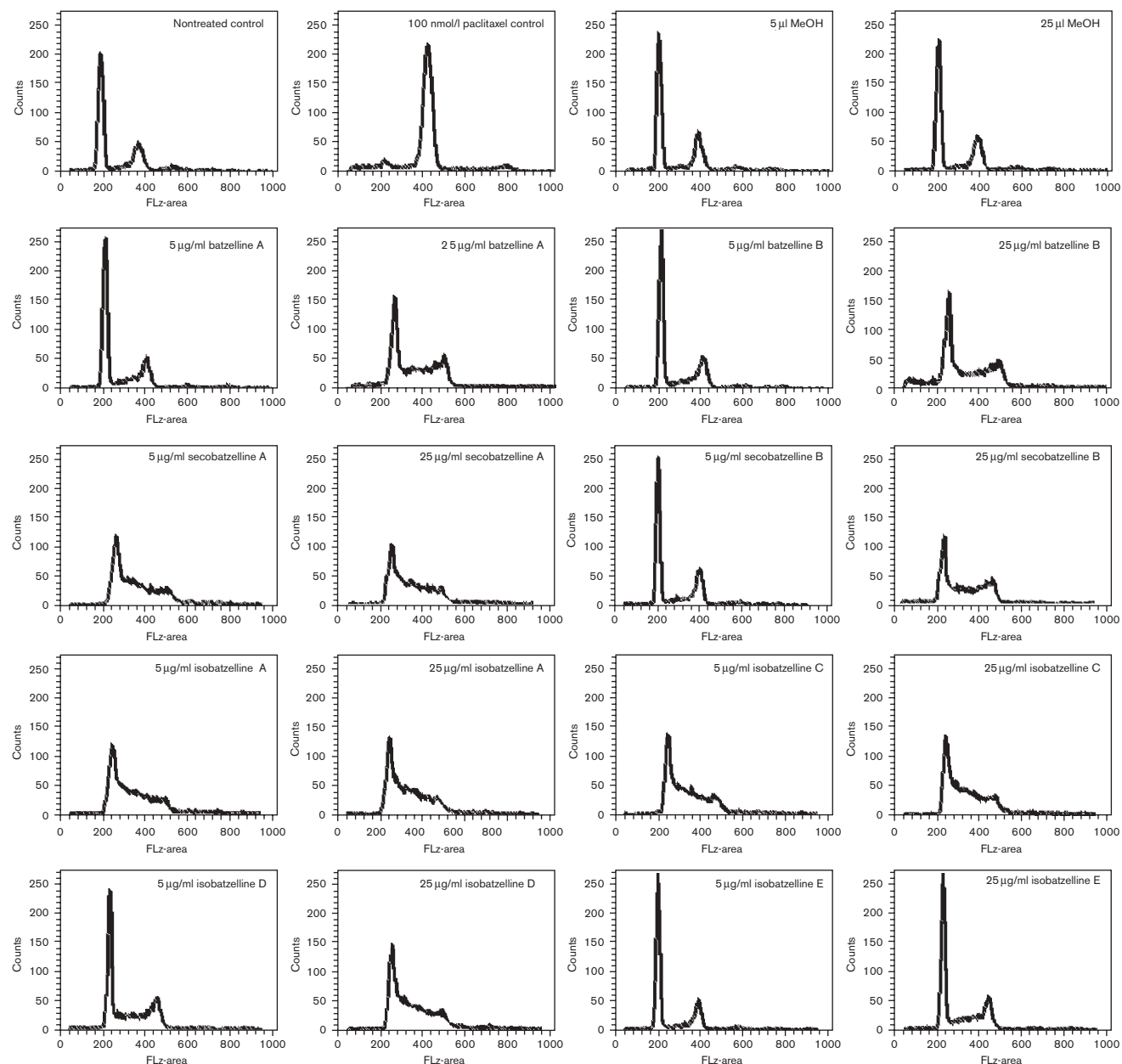
As topoisomerase II inhibition leads to cell cycle arrest [13], cell cycle analysis was performed on AsPC-1 cells treated with the batzellines to confirm our decatenation experiment. As shown in Fig. 3, at the dose of 25 µg/ml all of the batzellines caused cell cycle arrest, but only isobatzelline E gave us a very modest G2 arrest. The remainder of the batzellines tested caused cell cycle arrest at S phase suggesting that their mechanism of action is

**Fig. 2**



Isobatzelline E, batzelline B and secobatzelline A inhibit topoisomerase II activity. (a) Decatenation of kinetoplast DNA by topoisomerase II in the presence of the batzelline compounds, etoposide (positive control), methanol, or dimethylsulfoxide (vehicle controls) was visualized through electrophoresis. (b) Gels were subjected to densitometry analysis and the values were plotted using Microsoft Excel. Samples were tested once per experiment and the experiment was performed seven times. The graph shows the average of seven experiments  $\pm$  standard error of the mean ( $n = 7 \pm \text{SEM}$ ) and their statistical significance determined through the Student's *t*-test. DMSO, dimethyl sulfoxide.

Fig. 3



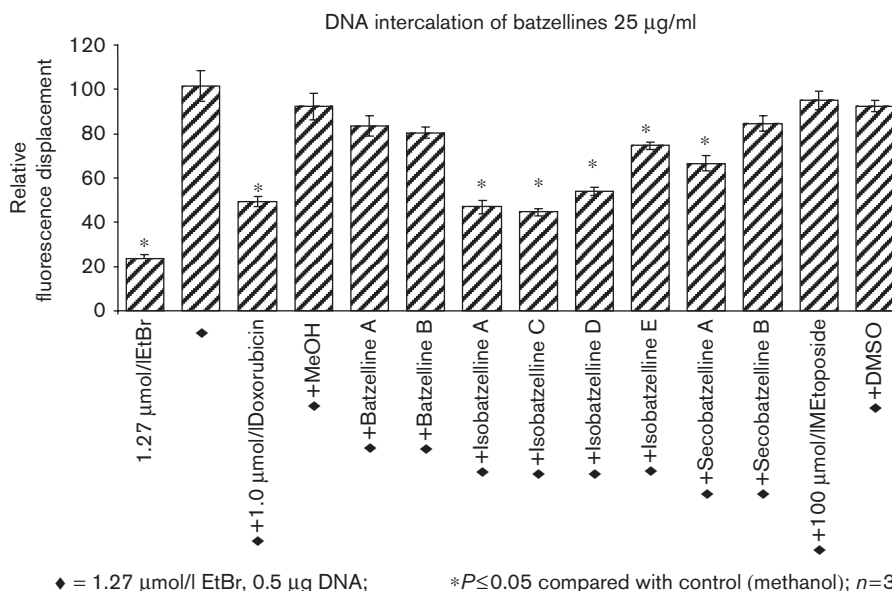
Treatment of AsPC-1 Cells with the batzellines induces cell cycle arrest. AsPC-1 cells were treated for 48 h in the presence of methanol (vehicle control), media alone, 5 or 25 µg/ml of the batzellines, or 100 nmol/l paclitaxel (positive control) and subjected to cell cycle analysis by flow cytometry. Samples were tested once per experiment and the experiment was performed in triplicate ( $n=3$ ). One representative experiment is shown.

through interfering with DNA synthesis. Treatment with 5 µg/ml resulted in an induction of S-phase cell cycle arrest by batzelline A, isobatzellines A, C, D, and secobatzelline A, whereas batzelline B, isobatzelline E, and secobatzelline B did not have an effect at this dose (data not shown).

The cell cycle analysis and the assay for topoisomerase II inhibition suggested the existence of a different mechanism of action, whereas the structure of the

batzellines suggested the strong possibility of they being DNA intercalators. Therefore, a fluorescence displacement assay was performed to test the ability of the batzellines to intercalate into DNA. Isobatzellines A, C, D and E, and secobatzelline A exhibited significant intercalation into DNA at both 5 and 25 µg/ml (Fig. 4). The best intercalators were isobatzelline A, C, and D. Isobatzelline E and secobatzelline A exhibited very modest intercalation.

Fig. 4



Isobatzelline A, C, D, E, and secobatzelline A have the ability to intercalate into DNA. Salmon testes DNA was allowed to interact with ethidium bromide for 30 min. At the end of this incubation, 5 or 25 µg/ml of the batzellines, methanol (vehicle control), 1 µmol/l doxorubicin (positive control), or 100 µmol/l etoposide were added to each reaction and allowed to interact for 30 min. The intercalation of ethidium bromide into DNA provides a fluorescent signal. Displacement of ethidium bromide by a DNA intercalator was detected by a decrease in the fluorescent intensity. The fluorescent signal was read in a plate reader under 340 nm excitation and 590 nm emission. Samples were tested once per experiment and the experiment was performed in triplicate ( $n=3$ ). The average of three experiments  $\pm$  standard deviation is shown. Statistical significance was determined through the Student's *t*-test. DMSO, dimethyl sulfoxide.

## Discussion

Similar to other marine pyrroloiminoquinones, the batzellines seem to act through more than one mechanism of action. The main mechanisms seem to be the inhibition of topoisomerase II and their ability to interfere with DNA synthesis by intercalating into DNA (Table 2). Interestingly, the most potent of the batzellines against pancreatic cancer cell lines seem to be those that act mainly through DNA intercalation. Thus, isobatzellines A, C, and D along with secobatzelline A show strong cytotoxicity against pancreatic cell lines with poor cytotoxicity against the Vero cell line. They all exhibit lower  $IC_{50}$ s than published values for gemcitabine for all cell lines except Panc-1 [14]. All of the batzellines have  $IC_{50}$ s below that observed for 5-fluorouracil, a chemotherapeutic drug currently used in the treatment of pancreatic cancer, in at least three of the cell lines used in this study, and are about three fold less potent than 5-fluorouracil in killing normal cells. This preferential cytotoxicity towards pancreatic cancer cells makes them interesting compounds. Both batzelline B and isobatzelline E seem to act mainly through their ability to inhibit topoisomerase II, although both have a very modest ability to intercalate into DNA. However, many known topoisomerase II inhibitors are also DNA intercalators [15]. Although they exhibit low cytotoxicity compared with the other batzellines, when given extra time to act (e.g. more than the 72 h at which our  $IC_{50}$ s are obtained),

they can effectively kill approximately 70% AsPC-1 at a dose of 25 µg/ml (Data not shown). Batzelline A and secobatzelline B are the least active members of the family in the assays used in this study. They induced little cytotoxicity and showed no DNA intercalation or topoisomerase II inhibition, although they were able to induce cell cycle arrest at S phase. Their mechanism for causing cell cycle arrest remains to be ascertained.

The structure of the batzellines predicted their ability to intercalate into DNA. However, no single functional group within the batzellines seems to be responsible for topoisomerase II inhibitory activity. The isobatzellines differ primarily in the side-chain substituents, yet although isobatzelline E is a very good topoisomerase II inhibitor, only isobatzelline A shows modest inhibitory activity whereas isobatzellines C and D show none. The second best inhibitor of topoisomerase II activity is secobatzelline A, whereas structurally related secobatzelline B shows no activity. Furthermore, no strong similarities exist between isobatzelline E and secobatzelline A. When compared with other marine pyrroloiminoquinones, both isobatzelline A and isobatzelline C are structurally similar to makaluvamine A, an excellent inhibitor of topoisomerase II activity [8], yet only isobatzelline A inhibits topoisomerase II. Isobatzelline E, our best inhibitor, is structurally similar to makaluvamine B, a poor topoisomerase II inhibitor [8].

The cytotoxic abilities of isobatzellines A, C, D, and secobatzelline A against pancreatic cancer cell lines along with their low toxicity against normal cells make them interesting compounds with potential chemotherapeutic effects that merit further research. In addition, although the limited availability of marine compounds is a major obstacle to their use as cancer therapeutics, syntheses of isobatzelline A and C have been reported [7], making their widespread use as chemotherapeutics a possibility.

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