Anticancer Activity of Novel pyrido[2,3-*b*]indolizine Derivatives: The Relevance of Phenolic Substituents

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Abstract. *Background/Aim: The potential of indolizine derivatives as anticancer agents has been shown through recent studies. Herein, we present our experimental results, showing that pyrido[2,3-b]indolizine derivatives are effective against colorectal cancer (CRC) cell lines. Materials and Methods: Several pyrido[2,3-b]indolizine derivatives were synthesized and their anticancer potential was evaluated against three CRC cell lines and two normal fibroblast cultures. Results: Our experiments identified 4-(3,4)-dihydroxyphenyl)-2-phenylpyrido[2,3-b]indolizine-10-carbonitrile (**4f**) as being active against all CRC cell lines at concentrations non-cytotoxic against fibroblast cultures. Additionally, cell-cycle analysis indicated that pyrido[2,3-b]indolizines can affect cell-cycle progression, with treated cells accumulating in the S- and G_2/M-phase. Conclusion: The hydroxyl groups in both the 3- and 4- positions of the aromatic substituent on C4 of the indolizine nucleus are crucial for activity against CRC cell lines. Further manipulation of the number and position of hydroxyl substituents on the aromatic rings may lead to improved anticancer activity of this class of compounds.*

Colorectal cancer (CRC) is the third most dominant cancer worldwide. CRC represents an increasing health risk with an estimated mortality rate of 600,000 in 2008 which is constantly rising (1). Approximately 25% of the patients show a familial history of the disease suggesting a contribution for shared genes and environment. The remaining 75%-80% of colorectal tumors are sporadic (2). Sporadic CRC cases can be divided into two sub-types. The first one is called the "canonical" (adenoma-carcinoma sequence) or "suppressor" pathway and involves chromosomal instability (CIN) and is often characterized by mutations in the genes encoding for KRAS and APC. The second type is the Microsatellite INstable tumors (MIN), which accounts for approximately 15%-20% of CRC cases. MIN tumors are characterized by microsatellite instability (MSI); often caused by *MLH1* promoter hypermethylation, and often show *BRAF* mutations (3, 4).

Current treatment for CRC is based on surgery, sometimes in combination with radiotherapy and/or chemotherapy. Systemic chemotherapy has been based on 5- fluorouracil, with the more recent introduction of other cytotoxic agents, such as irinotecan and oxaliplatin. Recently cetuximab and panitumumab, two monoclonal antibodies targeting the epidermal growth factor receptor, have proven to be effective in combination with chemotherapy or as single agents for treatment of CRC (5, 6). Despite advances in CRC treatment, the prognosis for patients with metastatic CRC remains poor, with a median overall survival of 18 to 21 months (5-8). The high patient burden of current chemotherapies and the lack of effective targeted therapies are compelling reasons to search for new (target) drugs against CRC.

Academia and pharmaceutical industries have shown interest in molecules incorporating the indolizine core due to the diverse biological applications of this scaffold. Natural and synthetic derivatives have been identified as anticancer, anti-inflammatory, anti-tuberculosis, anti-viral and anti-microbial agents (9-21). Considering the wide range of biological activities, indolizine derivatives are now being used as valuable leads for the design and synthesis of new biologically-active analogues.

In this context, our present work aimed to evaluate the biological activity of previously- and novel-synthesized indolizine derivative in colorectal cancer and normal cells.

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Key Words: Colorectal cancer, indolizine derivatives, cytotoxicity, HCT116, HT-29 and RKO cell lines, cell cycle.

Materials and Methods

Chemistry. All compounds were fully-characterized by elemental analysis and spectroscopic data. The NMR spectra were recorded on a varian Unity Plus at 300 MHz for 1 h and 75 MHz for ^{13}C or on a Bruker Avance 3400 at 400 MHz for ^{1}H and 100 MHz for ^{13}C, including the ^{1}H-^{13}C correlation spectra (HMQC and HMBC). Deuterated DMSO was used as solvent. The chemical shifts are expressed in δ (ppm) and the coupling constants (*J*) in hertz (Hz). The peak patterns are indicated as follows: s, Singlet; d, doublet; t, triplet; m, multiplet and br, broad. IR spectra were recorded on a FT-IR Bomem MB using Nujol mulls and NaCl cells. The reactions were monitored by thin-layer chromatography (TLC) using silica gel 60 F254 (Merck). The melting points were determined on a Stuart SMP3 melting point apparatus. Elemental analyses were performed on a LECO CHNS-932 instrument.

*Synthesis of pyrido[2,3-b]indolizine-10-carbonitrile **4**.* α,β-Unsaturated dicarbonyl compound **3** (0.21 mmol) was added to a solution of 1,1'-[2-amino-1-cyanoprop-1-ene-1,3-diyl]dipyridinium dichloride **2** (0.29 mmol) and sodium acetate (1.10 mmol) in EtOH (6 mL) and water (1 mL). The solution was re-fluxed for 3-7 days and then concentrated in the rotary evaporator. After cooling in an ice-bath, the solid started to precipitate. The solid was filtered and washed with water, leading to the pure product **4**.

Compounds **4a-g** were previously synthesized and reported (23) (^{1}H and ^{13}C NMR data available) and indolizines **4h-n** are novel compounds and were fully characterized in this publication. ^{1}H and ^{13}C NMR data are available upon request.

Viability experiments. Colorectal cancer cell lines HT-29, HCT116 and RKO were cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM Glutamax-I and 50 U/mL penicillin and 50 μg/mL streptomycin (GIBCO, Invitrogen LTD, Paisley, UK). Fibroblasts were maintained in DMEM/F-12 (1:1) supplemented with 10% FBS and 50 U/mL penicillin and 50 μg/mL streptomycin (GIBCO, Invitrogen LTD, Paisley, UK). All cell cultures were performed at 37˚C at 6% CO_2 in a humidified stove. Cell line identity was confirmed using the Promega Cell ID system (Promega, Madison, WI, USA).

Compounds were dissolved in 100% DMSO at 10 mM. Cells were seeded at a density of 12,500 cells/cm^2 24 h prior to compound addition. The cells were treated with the compounds for 72 h, after which cell viability was assessed using prestoBlue® assay (Invitrogen LTD, Paisley, UK) according to the manufacturer's specifications. In each experiment negative controls with and without 0.5% DMSO were included.

All viability assays were performed in triplicate in 96-well plates and reproduced in 2 independent experiments. The IC_{50} concentration was determined using Graphpad Prism® software (version 5.01).

Cell-cycle analysis. HCT116 cells were plated in T-25 flasks at a density of 12,500 cells/cm^2 24 h before incubation with test compounds. After an incubation period with test compounds of 72 h, cells were trypsinized, washed with PBS and fixed with methanol 90% at –20˚C for 30 min. After rinsing first with PBS/Tw 0.05% and then with PBA/Tw, the cells were incubated with staining solution (1% RNAse and 1mM of PI in PBS) at 37˚C for 30 min. Cell-cycle progression was analyzed by flow cytometry using a LSRII (BD Biosciences, Erembodegem, Belgium) flow cytometer counting 50,000 single cells per sample. Mathematical modeling and quantification of cell-cycle phases was performed using ModFit 3.2.1 and WinList 6.0 (Verity Software House, Inc., Topsham, ME, USA).

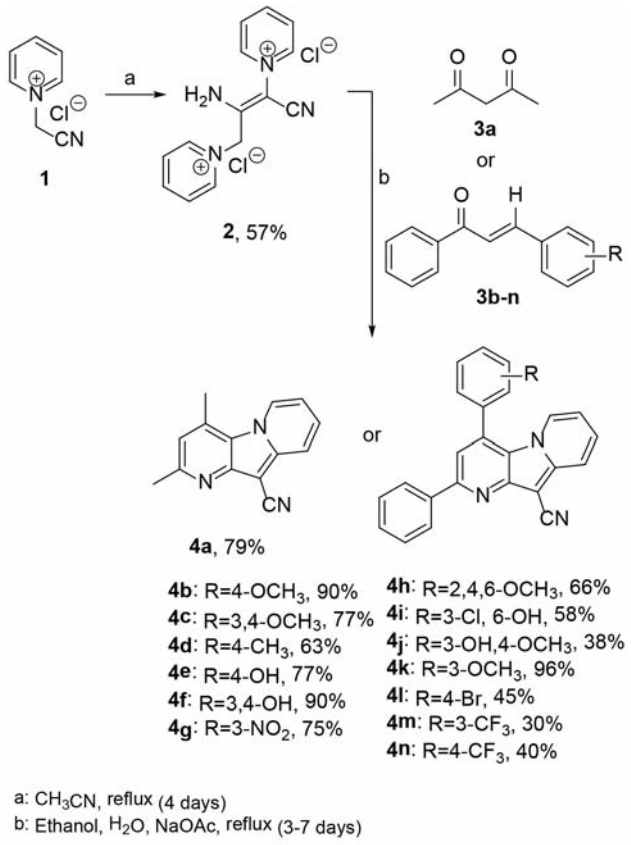

Figure 1. *Scheme of the synthetic pathway for the preparation of pyrido-indolizines 4.*

Results

Chemistry. In a previous study di-pyridinium salt **2** was generated from 1-(cyanomethyl)pyridinium chloride **1** after refluxing the starting material in acetonitrile for four days (Figure 1) (11). The reaction of acetylacetone **3a** and easily available α,β-unsaturated carbonyl compounds **3a-i** with the stable dimer **2** was studied using a mixture of ethanol and water, in the presence of sodium acetate and under reflux conditions. This efficient and simple one-pot procedure allowed the preparation of substituted pyrido[2,3-*b*]indolizine-10-carbonitriles **4a-g** (11) and was herein used to synthesize novel derivatives **4h-n**. The products were isolated by simple filtration in a high purity form and in moderate-to-excellent yields. All new compounds, fully characterized by the usual spectroscopic techniques, share a common substituted indolizine skeleton. Variations in the substitution pattern were only performed in the aromatic ring on C4 of the heterocyclic moiety.

Table I. *Cytotoxicity studies on HCT116, HT-29 and RKO cells using the prestoBlue® assaya.*

Comp.	HCT116		HT-29		RKO	
(μM)	25	50	25	50	25	50
4a	77±0	37±0	99±3	92±2	59±2	22±1
4b	96±12	95±15	100±3	100±2	90±2	83±0
4c	98±3	90±3	100±5	100±3	68±1	43±1
4d	96±1	87±7	100±4	100±3	73±1	61±2
4e	100±3	81±4	100±11	100±14	62±5	34±3
4f	20±2	2±0	48±2	11±2	12±1	1±10
4g	93±1	74±8	100±1	100±1	61±1	51±4
4h	90±11	87±8	100±5	100±9	43±2	32±2
4i	97±3	93±1	100±2	100±1	77±4	70±1
4j	82±3	58±3	86±9	53±2	40±0	20±2
4k	95±2	89±3	90±6	93±8	75±0	65±1
4l	98±2	84±6	100±2	100±4	69±2	48±4
4m	73±10	57±5	93±5	86±0	35±0	24±4
4n	99±3	94±1	100±9	99±4	87±4	81±1

[a]Data represent the percentage of viability after 72 h of compound treatment of two independent experiments ±error.

Table II. *Tumor-specific cytotoxic activity of selected compounds against CRC cell lines and normal cells.*

Comp.	Absolute IC_{50} (μM)					TSI[a]
	CRC cell lines			Normal cells		
	HCT116	HT-29	RKO	F-1	F-2	
4a	>50	>50	41.6	>50	>50	1.10
4f	14.8	14.3	14.2	34.6	34.7	2.40
4h	>50	>50	28.6	--- [b]	--- [b]	--- [b]
4j	>50	>50	23.4	--- [b]	--- [b]	--- [b]
4m	>50	>50	13.4	4.1	3.2	0.15

[a]TSI= [$\sum$ IC_{50} (normal cells)/$\sum$ IC_{50} (tumor cell lines) × (2/3)]. [b]not determinated.

Biological assays. The cytotoxicity of compounds **4a-n** was assessed against the CRC cell lines HT-29, HCT116 and RKO. This selection represents the three main sub-types of CRC. HCT116 originates from a hereditary CRC case; RKO and HT-29 represent sporadic CRC with and without MSI respectively.

An initial screening was performed at 25 μM and 50 μM in these three CRC cell lines (Table I). Growth inhibition was evaluated after 72 h of incubation using prestoBlue®.

The results indicate that, in general the indolizine derivatives displayed a higher cytotoxicity against RKO cells. Compound **4a** was moderately active and compounds **4f**, **4h**, **4j** and **4m** presented superior activity, with a cell viability below 50% at 25 μM. With the exception of **4a**, the remaining structures share a common scaffold of substituted indolizine where one of the aromatic rings is substituted by hydroxyl and/or methoxyl groups. Compound **4f**, with two hydroxyl groups in the phenolic substituent, was the most active molecule on RKO cells and was also active on HCT116 and HT-29 cell lines.

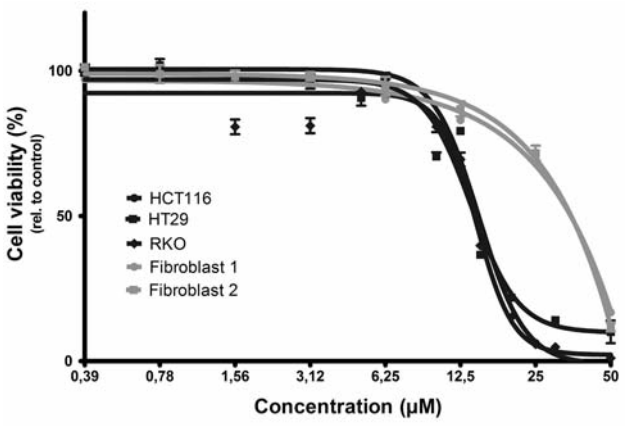

Figure 2. *Dose-response curves of CRC cell lines and fibroblast cultures treated with compounds **4f**.*

Based on these results, IC_{50} determinations were performed for these compounds on the same CRC cell lines. To assess cytotoxicity against normal cells, IC_{50} determinations were also performed on two fibroblast cultures (F-1 and F-2) for compounds **4a**, **4f** and **4m**. The dose-response curves for compound **4f** are shown in Figure 2. The results for all tested compounds are summarized in Table II. Cytotoxicity results including 95% confidence interval are available upon request.

To quantify the difference in effect on CRC cell lines compared to normal cells, the Tumor Specificity Index (TSI) was calculated for compounds **4a**, **4f** and **4m**, as described previously (23). For the compounds tested, only in the case of compound **4f** the tumor cell lines were more sensitive than the normal cells showing a TSI of 2.4 (Table II).

Compound **4f** was clearly the most active molecule and the presence of the hydroxyl groups in the 3- and 4- position of the aromatic ring may be responsible for the promising biological activity. A single hydroxyl group in the 4-position (**4e**) results in poor activity, but replacing it by a methoxyl group (**4b**) practically inactivates the compound. The 3-hydroxy-4-methoxy substituted derivative **4j** regains moderate activity, suggesting that the presence of oxygen atoms on both these positions, preferably linked to a proton, improves the activity.

Cell-cycle analysis was performed on HCT116 by flow cytometry for compounds **4a** and **4f** at IC_{50} concentrations. Cell-cycle distribution is graphically depicted in Figure 3. Compound **4f** showed a slight increase in S and G_2/M-phase populations relative to the DMSO control, and cells treated

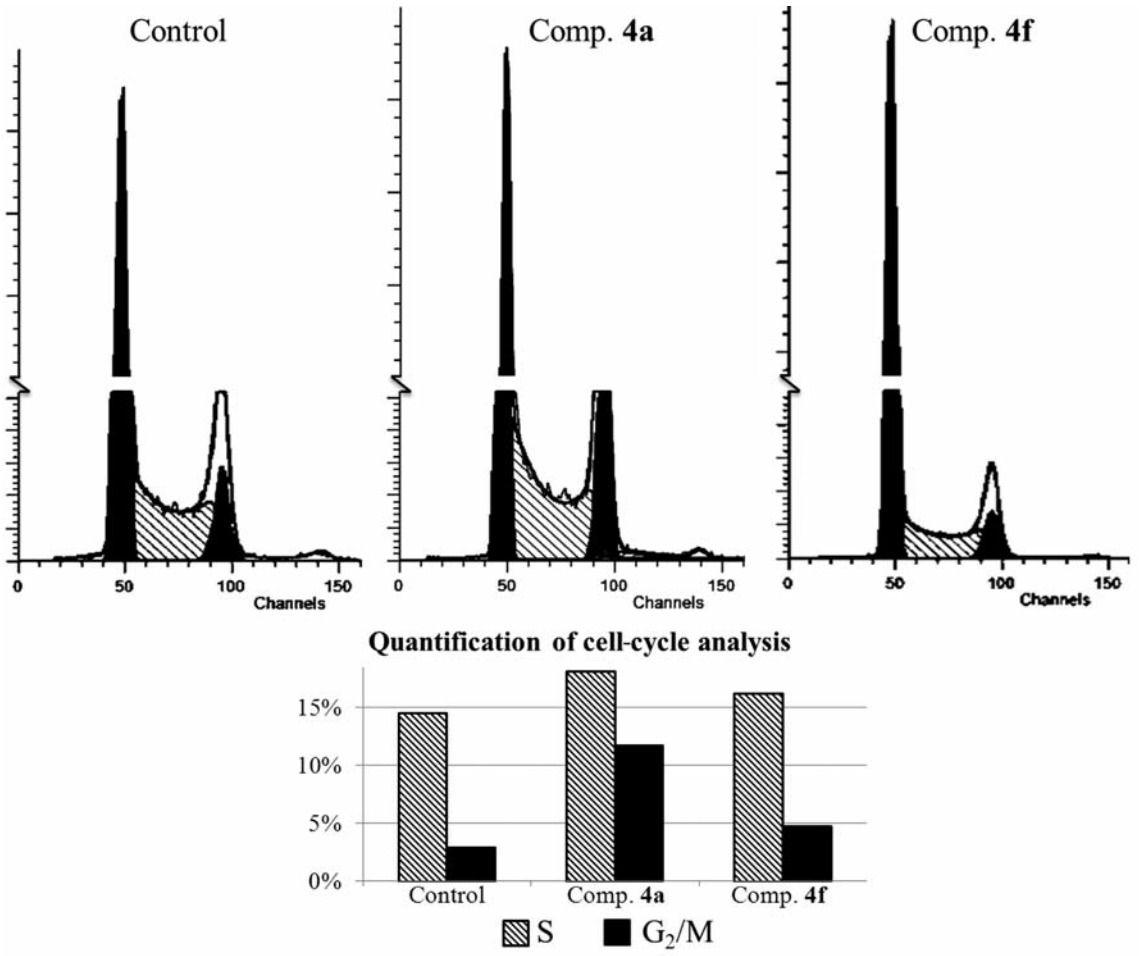

Figure 3. *Results of cell-cycle analysis using FACS analysis for compounds **4a** and **4f** and the DMSO control after 72 h of incubation at IC_{50} concentrations in HCT116. For each condition the 2 black peaks represent the G_1 and G_2/M phases, with the arced area in between representing the S-phase population. 3B: Quantification of S and G_2/M populations after compound treatment.*

with **4a** were found to have an increased percentage of cells in G_2/M phase. Quantification of these results showed that compounds **4f** resulted in a 12% increase in S-phase cells, accompanied by a 66% increase in G_2/M -phase. Compound **4a** was found to have a 25% increase in S-phase and G_2/M phase increased over 4-fold.

Discussion

In conclusion, the first round of anticancer screening showed compounds **4a**, **4f**, **4h**, **4j** and **4m** to be active against RKO. The remaining compounds were more effective against normal cells then against the CRC cell lines.

This could be explained by an alteration in the CRC cell lines which makes the cells resistant to these particular compounds. The cell-cycle analysis of cells treated with compound **4a** points towards an inhibition of the cell cycle during G_2/M -phase.

Compound **4f** was the only compound proven to be active against HT-29 and also HCT116. Cytotoxicity experiments using fibroblast cultures indicate that **4f** is not toxic at the concentrations required to reduce the viability of the CRC cell lines by 50%. Cell-cycle analysis of cells treated with this compound showed a modest shift in cell cycle, towards S and G_2/M phase. These effects of indolizine derivatives on cell cycle are consistent with a recent publication by Chaniyara *et al*. Herein, an increase in S-phase was also observed after 24 h of treatment with indolizine-like compounds, shifting towards a G_2/M after 48 h of compound treatment (21). In combination with our results showing a modest effect of pyrido-indolizine derivatives on cell-cycle progression, this suggests that indolizine derivatives are capable of influencing cell-cycle progression.

Our results warrant further investigation into compound **4f** to discover its precise mode of action. A structure-activity relationship study followed by RNA and protein analysis

needs to be performed to determine which structural properties of this specific indolizine derivative are at the basis of this molecular effect.

Our data suggest that the presence of two hydroxyl groups in the 3- and 4- positions of the aromatic substituent on C4 of the indolizine nucleus may be responsible for the superior activity of these derivatives. Further manipulation of the number and position of the hydroxyl groups on the aromatic rings may lead to improved activity, revealing the potential of these compounds as anticancer drug candidates.

Acknowledgements

We gratefully acknowledge the financial support from University of Minho and FCT through the Portuguese NMR network (RNRMN), the Project F-COMP-01-0124-FEDER-022716 (ref. FCT PEst-C/QUI/UI0686/2011) FEDER-COMPETE and BPD grant awarded to Marta Costa (SFRH/BPD/79609/2011). We thank Jaap van Eendenburg for excellent support with cell cultures.

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Received December 20, 2013
Revised January 8, 2014
Accepted January 10, 2014