Synthesis and biological evaluation of novel oxophenylarcyriaflavins as potential anticancer agents†

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We report the synthesis and biological evaluation of new oxophenylarcyriaflavins designed as potential anticancer agents. An efficient synthesis involving palladium-catalyzed Suzuki and Stille reactions is presented, without any indolic protective group. The central ring closure of the scaffold was performed through an electrophilic reaction on the position *C*-2 of the indole ring. The use of indole and 5-benzyloxyindole, along with substituted phenyl rings, generated three different scaffolds, which were successively exploited to modulate the structure. The cytotoxicity of the newly designed compounds on four cancer cell lines and activities against three kinases (CDK1, CDK5 and GSK3) were evaluated. Several compounds showed a marked cytotoxicity with IC_{50} values in the sub-micromolar range, and induced important cell cycle perturbations, with a G2/M arrest. Some compounds revealed DNA binding properties and were found to inhibit topoisomerase-mediated DNA relaxation of supercoiled DNA, but these properties are not mandatory for a cytotoxic action. A novel lead compound (**32**) has been identified and warrants further investigations.

Introduction

Indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole alkaloids form a class of compounds endowed with potent antitumor, antiviral and/or antimicrobial activities.[1] This family has raised considerable attention because of the central role of these molecules in the regulation of cell cycle progression and specific enzyme inhibition.[2,3] Structure–activity relationships (SAR) in the indolocarbazole series have been extensively studied in the context of topoisomerase I inhibition and tumor cell killing. Compounds bearing a pyrroloindolocarbazole and possessing one *N*-glycosidic bond, such as the antibiotic rebeccamycin, generally function as DNA topoisomerase I inhibitors.[4] A few analogues such as NB-506 and J-107088 (also known as Edotecarin) have entered clinical trials for cancer treatment.[5–7] More recently, fluorinated derivatives of such molecules have been reported and their topoisomerase I-dependent anticancer activity looks promising.[8–10] We have recently described the bioisosteric replacement of an indole moiety by a 7-azaindole unit to afford the first symmetrical and dissymmetrical 7-azaindolocarbazoles **I** and **II** (Fig. 1).[11] In the same vein, the cytotoxic properties of the *N*-glycosylated derivatives of **I** and **II** have been reported by others.[12,13] For these different molecules, the role of topoisomerase I inhibition in the cytotoxic action seems relatively minor when compared to the NB-506-type series. The data suggested that other signalling proteins, particularly kinases, may play a role.

In order to develop selective kinase inhibitors, fitting in the ATP binding site, modifications of the aromatic heterocyclic indolocarbazole moiety appeared as a valid alternative to the synthesis of glycosylated compounds. In addition, most of the aryl carbazoles designed so far for kinase inhibition possess an unsubstituted maleimide whereas in general the most cytotoxic compounds bear hydrophilic side chains. It is noteworthy to mention also that closely related heteroarylcarbazoles (type **III**) have been described as inhibitors of Cyclin D1/CDK4.[14,15] Cyclin dependent kinases (CDKs) were also targeted with indolocarbazoles such as bis *N*-indolyl alkylated arcyriaflavins recently described as CDK1, CDK2 and CDK4 inhibitors.[16,17] In the NH maleimide series, indole *versus* (hetero)arylcarbazole replacement represents also an interesting strategy.

With this in mind, we envisaged the synthesis of different naphthalenic compounds **IV**.[18] The replacement of one of the two indoles by a naphthalene ring produced highly cytotoxic naphthocarbazoles **IV**, suggesting that the naphthalene is effectively a suitable bio-isostere for indole. At the same time, we developed a phenylcarbazole series **V** in order to broaden our SAR knowledge.[19] In both series, the SAR studies showed that i) for the phenylcarbazoles of type **V**, unsubstituted maleimide compounds were non cytotoxic and enhanced the inhibition of CDK, ii) the presence of a basic side chain on the maleimide led to cytotoxic agents with weak CDK inhibition, iii) the introduction of a hydroxyl group on the indole in position 5 greatly influenced the biological properties.

We have identified a few studies on bis-indolic compounds such as homoarcyriaflavin **VI**, arcyriacyanin A **VII** which were synthesized but as yet no significant activities have been reported.[20,21] In

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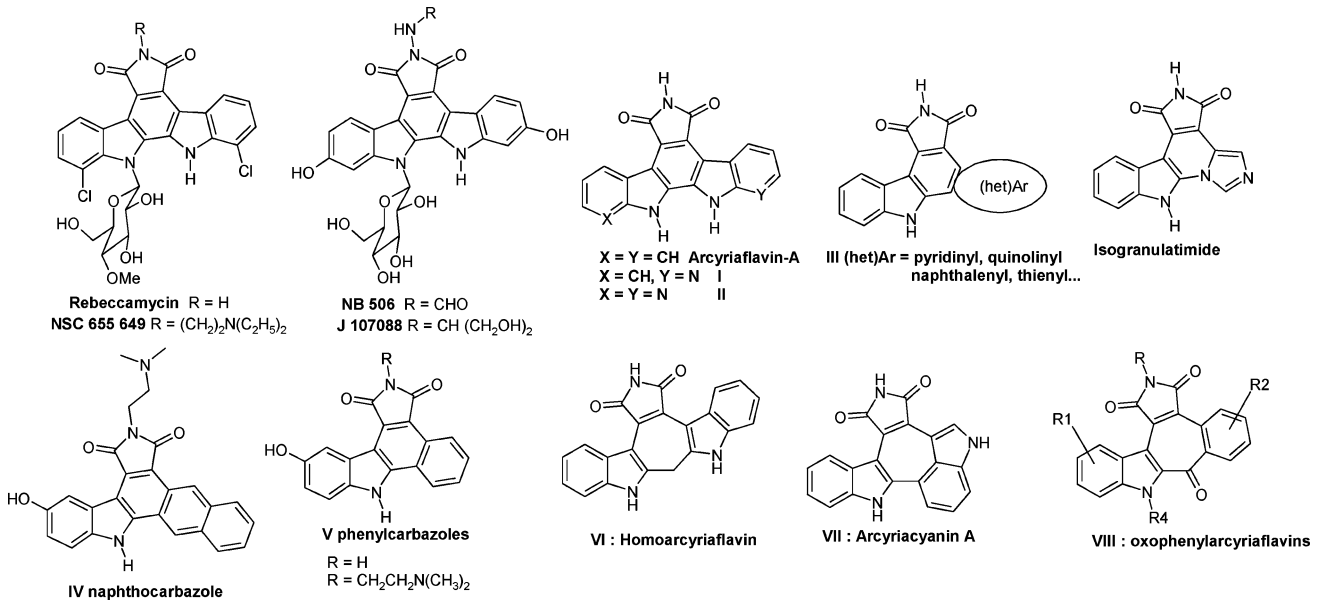
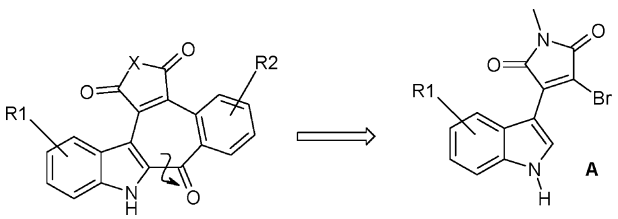


Fig. 1 Modifications of the indolocarbazole skeleton.

the present article, we report the design and the synthesis of new hybrid derivatives type **VIII** containing: an indole, an unusual seven-membered ring and a fused phenyl group. The central tropone moiety in **VIII** was surmounted by a fused substituted maleimide ring. In addition, we introduced molecular diversity by using indole or 5-hydroxyindole for the left part of the molecule and phenyl or dihydroxyphenyl rings for the right part to improve the SAR in this new series.

Chemistry

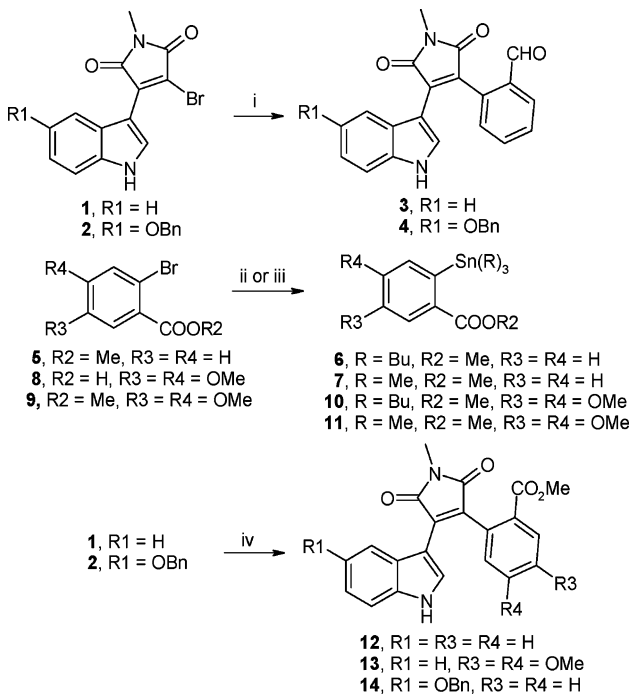
In this paper we report an improvement on the previously reported methodology[22] and a general synthesis (Scheme 1), which starts from indoles and involves (i) the introduction of the maleimide leading to **A**, (ii) a Suzuki or Stille cross coupling reaction adding the 2-formylphenyl or the 2-carboxyphenyl groups, (iii) the transformation of the aldehyde or the ester into carboxylic acid, iv) an intramolecular electrophilic cyclization to afford the attempted cycloheptatrienones (tropones) currently named oxophenylarcyriaflavins.



Scheme 1 Retrosynthesis of oxophenyl-arcyriaflavins.

A. Palladium catalyzed reactions

Following this strategy, we began the synthesis by preparing compounds **1** and **2**, classically obtained from indole or 5-benzyloxyindole and 2,3-dibromo-*N*-methylmaleimide in the presence of LiHMDS in THF in a fairly good yield.[18] First, a palladium-catalyzed cross coupling Suzuki type reaction between **1** and the commercially available 2-formylphenylboronic acid led to **3** (Scheme 2). The reaction was carried out in the presence of K_2CO_3 in a refluxing mixture of dioxane–water using $Pd(OAc)_2$ as catalyst. After 6 h, compound **3** was obtained in the best yield



Scheme 2 *Reagents and conditions*: i) 2-formylphenylboronic acid (1.5 eq.), $Pd(OAc)_2$ (0.1 eq.), K_2CO_3 (1.8 eq.), dioxane–water 85 : 15, rflx, from **1** to **3**, 6 h, 72%, from **2** to **4**, 3.5 h, 79%; ii) $Pd(PPh_3)_4$ (0.1 eq.), toluene, rflx, from **5** to **6**, Sn_2Bu_6 (1.2 eq.), 23 h, 47%, from **5** to **7**, Sn_2Me_6 (1.2 eq.), 2 h, 97%, from **9** to **11**, $Pd(PPh_3)_4$ (0.2 eq.), Sn_2Me_6 (1.5 eq.), 6 h, 81%; iii) from **9** to **10**, Sn_2Bu_6 (1.2 eq.), $PdCl_2(PPh_3)_2$ (0.1 eq.), LiCl (0.5 eq.), toluene, rflx, 24 h, 54%; iv) $PdCl_2(PPh_3)_2$ (0.2 eq.), CuI (0.1 eq.), dioxane, rflx, from **1** and **6** (1.5 eq.) to **12**, 6 h, 74%, from **1** and **10** (1.5 eq.) to **13**, 5.5 h, 78%, from **2** and **6** (1.5 eq.) to **14**, 3 h 45 min, 75%.

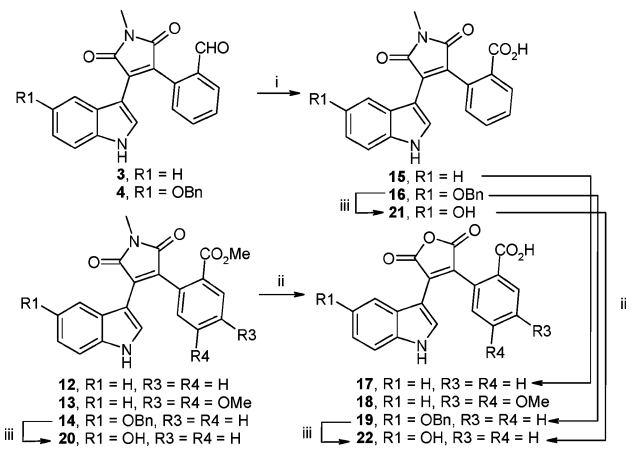
of 72%.[18,22] Applying these conditions to the 5-benzyloxyindole derivative **2** led to the desired compound **4** in a 79% yield.

An alternative method to insert a carbonyl group between the indole and the phenyl rings consisted in the direct introduction of the 2-carboxymethylphenyl unit using **5**, **8** and **9** as starting materials. We have previously prepared **6** and **7**.[22–24] By increasing the amount of $Pd(PPh_3)_4$ and Sn_2Me_6 we obtained **11** from **9** in an 81% yield. The compound **10** was obtained from **9** in best yield (54%) by modifying the nature of the palladium catalyst ($PdCl_2(PPh_3)_2$) and adding LiCl.

The Stille procedure applied to the bromo compound **1** and stannylated derivative **6** (1.5 eq.) was next performed with $PdCl_2(PPh_3)_2$ and CuI in refluxing dioxane. After 6 h, the desired compound **12** was obtained in 74% yield. Under similar conditions, the trimethyltin derivative **7** led to the same product **12** after 5 h in a 70% yield, and **10** afforded **13** in a 78% yield. Disappointingly, the trimethylstannyl derivative **11** led to **13** in only 53% yield in the best case. From **2** and **6**, the desired compound **14** was isolated without any difficulty in a fairly good yield (75%).

B. Synthesis of the carboxylic acids

The next step was aimed at generating a *C*-2 indolic keto function by oxidation of the aldehydes **3** (Scheme 3). This reaction was carried out with an aqueous solution of sodium chlorite and sulfamic acid at 6 °C, by accurate control of the temperature during the oxidant addition, and led to the acid **15** in a 81% yield.[22] Applying these conditions to compound **4** led to **16** in only a 59% yield. Lowering the temperature to 2 °C (limits of freeze) afforded **16** in 83% yield.



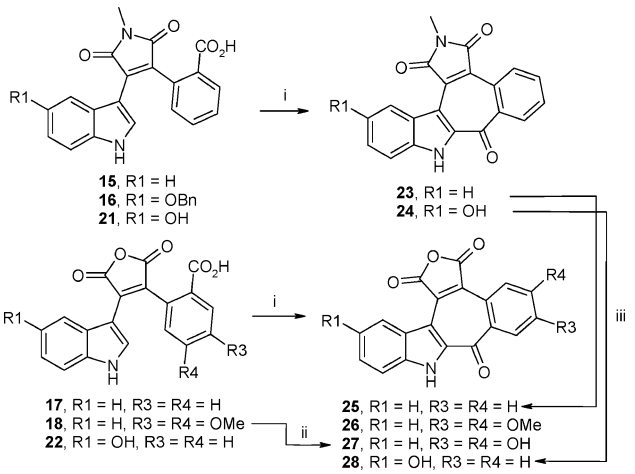
Scheme 3 *Reagents and conditions*: i) NH_2SO_3H (8.1 eq.), $NaClO_2$ (2.2 eq.), dioxane–water, 1 min, from **3** to **15**, 6 °C, 81%, from **4** to **16**, 2 °C, 83%; ii) a) aq. KOH (45%, 60 eq.), acetone, rt, b) HCl conc. to pH = 1, rt; from **12** to **17**, a) 20 h, b) overnight, quant., from **13** to **18**, a) 2 h, reflux and b) overnight, quant., from **14** to **19**, a) KOH (40 eq.), 7 h and b) overnight, 36%, from **15** to **17**, a) KOH (40 eq.), 28 h, and b) 3 min, 91%, from **16** to **19**, a) 24 h, and b) 24 h, 95%, from **21** to **22**, a) KOH (40 eq.), 25 h and b) 3 days, 94%; iii) BBr_3 (25 eq.), CH_2Cl_2, 0 °C to rt, 5 min, from **14** to **20**, 86%, from **19** to **22**, quant., from **16** to **21**, 83%.

The second method used was hydrolysis of the ester **12** using a large excess of an aqueous KOH solution in acetone for 20 h at room temperature. Then, acidification with a concentrated hydrochloric acid solution (pH = 1) led, after a few additional hours, to the maleic anhydride compound **17** in a quantitative yield. Starting from **13** and **14**, this reaction required adaptation of reaction times but yielded **18** and **19** in average to excellent yields.

In parallel, we cleaved the benzyl group of **14**, **16** and **19** using BBr_3 at room temperature to generate respectively compounds **20**, **21** and **22** in very good yields. Basic treatments of compounds **15**, **16** and **21** led to **17**, **19** and **22** respectively in satisfying yields.

C. Electrophilic intramolecular reaction

The ring closure leading to the central seven-membered cycle through an electrophilic cyclization without any pre-activation of the carboxylic acid function, was the next goal. Based on our knowledge, mild conditions involving a large excess of $BF_3{\cdot}Et_2O$ in refluxing DCE had to be applied.[22] After a few hours, the maleimide containing compounds **15** or **21** led to compound **23** and **24** in 89% and 60% yield, respectively. The same reaction carried out starting from **16** led to an intractable mixture. The benzyloxy group proved very sensitive toward Lewis acids (Scheme 4).



Scheme 4 *Reagents and conditions*: i) BF_3. Et_2O (40 eq.), DCE, rflx, from **15** to **23**, 5.5 h, 89%, from **21** to **24**, 2 h, 60%, from **17** to **25**, 20 h, 70%, from **18** to **26**, 5.5 h, 32%, from **22** to **28**, 19 h, 60% ii) BBr_3 (25 eq.), DCE, 0 °C to rflx, 47 h, from **18** to **27**, 88% iii) a) aq. KOH (45%, 40 eq.), acetone, rt, b) HCl conc. to pH = 1, rt, from **23** to **25**, a) 24 h, b) 12 h (overnight), quant., from **24** to **28**, a) 23 h and b) 2 days, 90%.

The intramolecular reaction performed with the anhydride **17**, required 20 h of reaction time to improve the yield of **25** to 70%. Compound **22** led to impure **28** in only 60% (as judged by ^1H NMR). Starting from **18**, the reaction led, even after flash chromatography to a mixture of compounds but a supplementary recrystallization afforded only **26** in a 32% yield.

We next attempted to selectively demethylate **18** with BBr_3 in dichloromethane. In all cases, a concomitant electrophilic cyclization, leading to complex mixtures, occurred. Thus, we set out for one pot cleavage and intramolecular reaction using a large excess of BBr_3 (25 eq.). At room temperature, after 18 h, only 25% of the desired product **27** was isolated. Increasing the temperature to reflux of dichloroethane led to the targeted compound in a good yield (88%) after 47 h. Disappointed by the problems associated with preparation of **28** from **22**, we decided to treat *N*-methylmaleimide **24** with alcoholic KOH solution for 23 h.

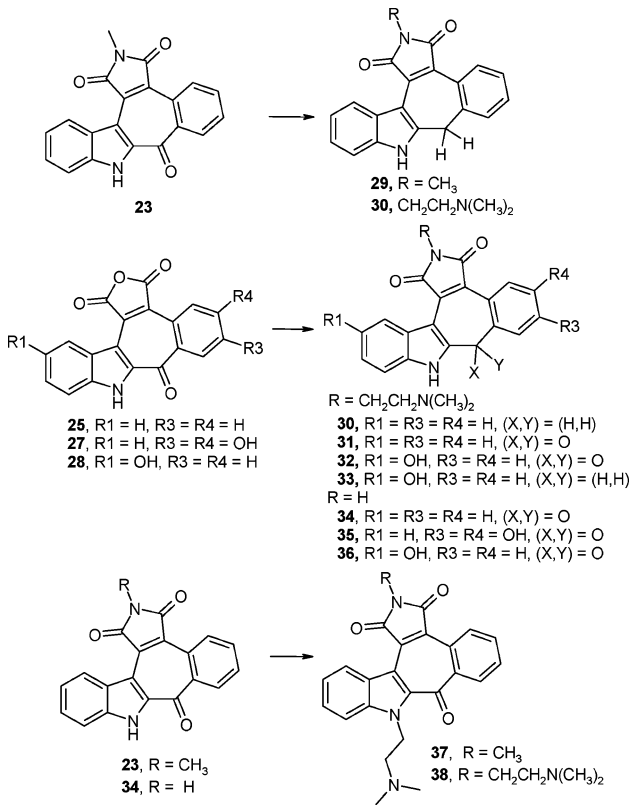
After the acidification step the attempted compound **28** was obtained in a 90% yield. Similarly, compound **23** furnished the derivative **25** in a quantitative yield.

D. Synthesis of functionalized oxophenylarcyriaflavins

The first modification we attempted was the introduction of a dimethylaminoethyl side chain on the maleimide or maleic anhydride part of scaffold **23**, **25**, **27**, **28** (Scheme 5, Table 1, entries 1–10). Previously, we reported that, in boiling dimethylaminoethylamine, in a sealed tube and in the presence of DMF, the reaction of compound **23** led only to the reduced compound **29** in 41% yield.[22] Under similar conditions, the reaction of the more reactive anhydride **25** afforded compound **31** in 59% yield (entries 1,2). Application of these reaction conditions to our new derivatives **27** and **28** was fruitless. These results prompted us to investigate other conditions. In open glassware, in boiling dimethylaminoethylamine, compound **30** was synthesized either from maleimide **23** or from anhydride **25** in 48% and 86% yield, respectively (entries 3, 4). All attempts starting from **27** failed again.

In order to reduce the reaction time, we next investigated the reactivity of **25**, **27** and **28** in the presence of dimethylaminoethylamine under microwave irradiation (Biotage apparatus).[25] In the above diamine, at 150 °C, compound **25** led to the desired compound **31** after 15 min, but in only 10% yield. It is possible that the poor solubility of **25** causes the low yield. Next, the two reagents were adsorbed on a solid phase.[26] From the furo derivatives **25** and **28**, the direct absorption on silica gel with 5.0 eq. of diamine led to the attempted products **31** and **32** in an improved yield of 48% and 33% respectively. Starting materials (30%) and reduced compounds **30** or **33** were also isolated during the purification steps (entries 5, 6).

The next attempted variation dealt with the reaction of **25** with ammonia in the presence of DMF (entry 7).[19] Refluxing for 67 h an aqueous solution of NH_4OH (30%) containing **25** led to compound **34** in 70% yield with no trace of reduction of the tropone. Another solution to perform the NH insertion consisted in the reaction of the anhydride **25** with melted NH_4OAc. After 2 h, the derivative **34** was isolated in a best yield of 81%. The major advantage of this method was its compatibility with the presence of quinonic or quinonimine sensitive systems. So, we next performed the reaction with the two anhydrides **27** and **28** to isolate compounds **35** and **36** in 65 and 73% yield, respectively (entries 8–10).



Scheme 5 For reagents and conditions see Table 1.

Then, we used compounds **23** and **34** to introduce the dimethylaminoethyl side chain onto the free indolic nitrogen atom (entries 11, 12) in the presence of NaH and chloroethyldimethylamine in DMF at 90 °C. From compound **23**, the derivative **37** was isolated in a 84% yield, whereas starting from **34**, both nitrogen atoms reacted with the chloroalkyl derivative and the compounds **31** and

Table 1 Substitution of derivatives, **23**, **25**, **27**, **28** and **34**

Entry	Starting material	Reagent	Conditions	Temperature	Time	Product (yield)
1	**23**	Excess of $NH_2(CH_2)_2N(CH_3)_2$	Sealed tube, DMF	rflx	22 h	**29** (41%)[b]
2	**25**	Excess of $NH_2(CH_2)_2N(CH_3)_2$	Sealed tube, DMF	rflx	18 h	**31** (59%)[b]
3	**23**	Excess of $NH_2(CH_2)_2N(CH_3)_2$	Open flask without solvent	rflx	22 h	**30** (48%)[b]
4	**25**	Excess of $NH_2(CH_2)_2N(CH_3)_2$	Open flask without solvent	rflx	64 h	**30** (86%)
5	**25**	$NH_2(CH_2)_2N(CH_3)_2$ (5 eq.)	μwave, SiO_2 (8.5 eq.)	150 °C	15 min	**31** (48%)[b], **30** (15%)[a]
6	**28**	$NH_2(CH_2)_2N(CH_3)_2$ (5 eq.)	μwave, SiO_2 (8.5 eq.)	150 °C		**32** (33%)[b], **33** (9%)[a]
7	**25**	Aqueous NH_4OH	DMF	rflx	67 h	**34** (70%)[b]
8	**25**	Fused NH_4OAc in excess		130 °C	2 h	**34** (81%)[b]
9	**27**	Fused NH_4OAc in excess		130 °C	3 h	**35** (65%)[b]
10	**28**	Fused NH_4OAc in excess		130 °C	3 h 30 min	**36** (73%)[b]
11	**23**	$ClCH_2CH_2N(CH_3)_2$ (2.5 eq.)	NaH (3.8 eq.), DMF	90 °C	9 h	**37** (84%)[b]
12	**34**	$ClCH_2CH_2N(CH_3)_2$ (2.5 eq.)	NaH (3.8 eq.), DMF	90 °C	24 h	**31** (13%)[b], **38** (30%)[b]
13	**34**	$ClCH_2CH_2N(CH_3)_2$ (2.5 eq.)	K_2CO_3	65 °C	24 h	**31** (33%)[b], **38** (10%)[b]

[a] Estimated by ^1H NMR. [b] Isolated product.

38 were obtained in 13 and 30% yield respectively. Replacing NaH for K_2CO_3 and performing the reaction at 65 °C slightly favored the maleimide substitution and compound **31** was isolated in 33% yield whereas only 10% of **38** was obtained (entry 13).

Biological results and discussion

The newly synthesized compounds were tested for DNA binding and topoisomerase inhibition. Their cytotoxic properties were also measured. The data are collected in Table 2. DNA interaction was first estimated using a conventional melting temperature assay with calf thymus DNA (CT-DNA, 42% GC). Unsurprisingly, the molecules bearing a dimethylaminoethyl side chain were found to stabilize CT-DNA whereas the other neutral molecules showed little, if any, interaction with nucleic acids. Compounds **31** and **32** bearing the cationic chain on top of the maleimide ring showed marked interaction with DNA, with ΔT_m of 7 °C and these two compounds were markedly cytotoxic toward HL60 human leukemia cells. Compound **32** showed the most cytotoxic activity in the series, with an IC_{50} of 0.3 μM close to that of the reference antitumor antibiotic rebeccamycin. The hydroxyl group on the indole ring contributed modestly to DNA binding but, more significantly, to cytotoxicity (and solubility as well). Interestingly, by comparing compounds **30** and **31**, as well as **32** and **33**, it clearly appeared that the reduction of the keto group of the central tropone ring strongly reduced the DNA binding capacity and abolished the cytotoxic effects, suggesting that DNA interaction may contribute (to some extent) to the biological activity of **31** and **32**. This conclusion is also supported by the observation that the other neutral compounds that bind weakly, or with no detectable binding to DNA, correspondingly showed no significant cytotoxic properties. However, DNA interaction is not sufficient to determine cytotoxicity since compound **38**, with two dimethylaminoethyl side chains on the maleimide and indole rings, was found to be poorly cytotoxic towards HL60 cells despite its superior propensity to DNA binding.

DNA binding of four key molecules (**30–32**, **38**) was investigated further using the alternative polymer poly(dAdT)$_2$. Under the experimental conditions used (16 mM Na^+), the helix-to-coil transition occurs at 42 °C in the absence of drugs. The T_m was shifted to higher temperatures with the four drugs and more pronounced with the keto derivative **31** as compared to the methylene analogue **30** (Fig. 2). Thus corroborating the previous observation with CT-DNA, we can conclude that the keto group is directly involved in DNA recognition (through H-bonding, for example) and/or indirectly, by providing a more planar chromophore. The ΔT_m values reached 18 °C with compound **38**, and the molecules rank order **38** > **31**, **32** > **30**.

The mode of binding to DNA was investigated by spectroscopic methods. These compounds intercalate into DNA and this is particularly clear with compound **38**. DNA induces significant shifts in the UV–Visible spectra of **38** with a marked decrease in the extinction coefficient around 450 nm and a shift of the band to a much longer wavelength (Fig. 3). The UV–Visible spectral changes are more subtle with **32**. Circular dichroism (CD) measurements showed that a negative band centered at 350 nm appeared upon addition of CT-DNA. This band reflects the orientation of the tetracyclic chromophore bound to the double helix and is entirely consistent with an intercalative binding process. Intercalating

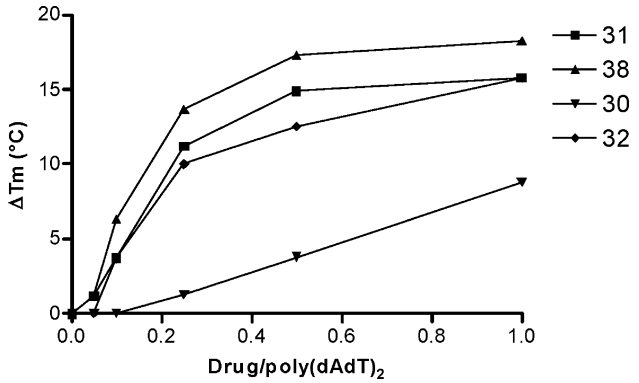


Fig. 2 Melting temperature variation ΔT_m (T_m drug-DNA complex − T_m DNA alone in °C) of poly(dAdT)$_2$ after incubation with drugs at increasing drug/DNA. T_m measurements were performed in BPE buffer, in 1 cm quartz cuvettes at 260 nm with a heating rate of 1 °C min^{-1}. The T_m values were obtained from first-derivative plots.

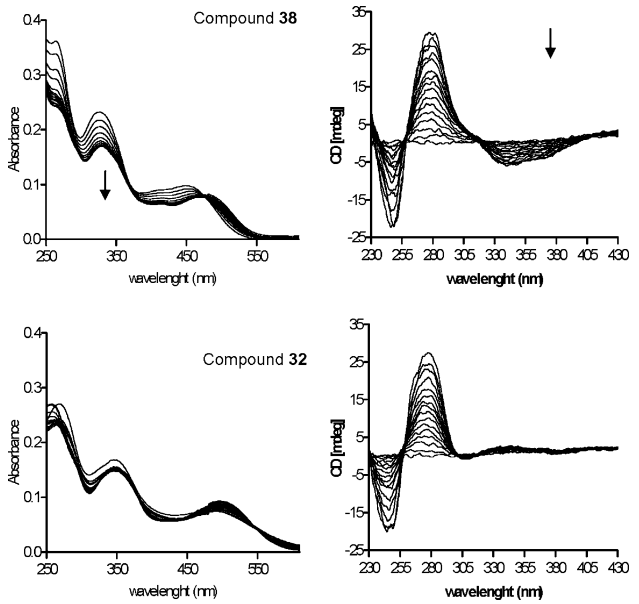
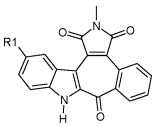
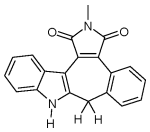
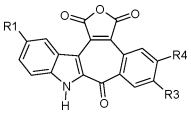
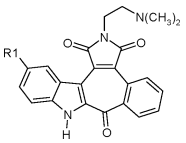
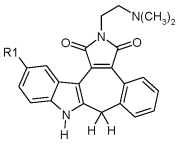
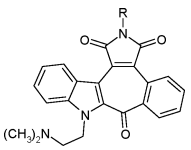
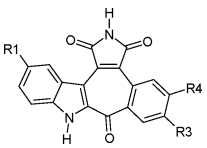


Fig. 3 (left) Absorption and (right) CD DNA titration of compound **38** and **32** in BPE buffer for absorption and cacodylate buffer for CD DNA titration. Aliquots of a concentrated calf thymus DNA solution were added to 1 ml of a drug solution (20 μM for the absorbance and 50 μM for the CD measurements). The drug/DNA ratios increased from 0 to 1.

agents usually (but not always) give this type of negative CD signal whereas positive signals are commonly seen with minor groove binders. The negative CD band was not observed with **32** but the unwinding data (see below) also support intercalation.

Based on the structural analogy with rebeccamycin, the compounds were tested as potential inhibitors of topoisomerases I and II. In all cases, none of the compound was found to act as a "poison" capable of stabilizing topoisomerase–DNA complexes, as can be commonly detected with the reference drugs camptothecin (for topoisomerase I) or etoposide (for topoisomerase II) (data not shown). An interference with the catalytic activity of the enzyme was observed in some cases, but this only reflects the DNA-binding capacity. As shown in Fig. 4, concentration-dependent inhibition of the relaxation of a supercoiled plasmid DNA with topoisomerase I was detected

Table 2 *In vitro* cytotoxic effect IC_{50} (μM); Fibro = human diploid skin fibroblastic cells (normal cell line); HL60 human leukemia cells (tumor cell line), Caco2 = human colon carcinoma cells (tumor cell line);[27] Huh7 = human hepatocarcinoma cells (tumor cell line);[28] F1 = RLEC for rat biliary epithelial cells clone F1 (tumor cell line)[29]

Compound		ΔT_m[a]	Fibro[c]	HL60[b]	Caco[c]	Huh7[c]	F1[c]
	**23** R1 = H	0	>25	>100	10	>25	10
	24 R1 = OH	0	10	27	2	30	20
	**29**	ND	>25	ND	20	4	4
	**25** R1 = R3 = R4 = H	0	>25	52	2	>25	0.2
	26 R1 = H, R3 = R4 = OMe	0	10	50	10	5	10
	27 R1 = H, R3 = R4 = OH	0	>25	>100	>25	>25	0.4
	28 R1 = OH, R3 = R4 = H	0	>25	>100	>25	>25	>25
	**31** R1 = H	6.8	10	1.22	1	2	0.4
	32 R1 = OH	7.1	0.6	0.31	0.5	1	0.07
	**30** R1 = H	2.5	10	72	3	30	0.7
	33 R1 = OH	ND	>100	ND	30	10	4
	**37** R = CH_3	ND	10	ND	10	2	2
	38 R = $CH_2CH_2N(CH_3)_2$	9.9	>25	17	7	6	2
	**34** R1 = R3 = R4 = H	1.2	10	22	0.3	>25	20
	35 R1 = H, R3 = R4 = OH	0.9	>25	5.81	10	0.3	2
	36 R1 = OH, R3 = R4 = H	0	>25	37	>25	10	20
References	Roscovitin	ND	20	ND	2	3	5
	Rebeccamycin	ND	ND	0.16	ND	ND	ND

ND = not determined.[a] Variations in melting temperature, $\Delta T_m = T_m^{\text{drug-DNA complex}} - T_m^{\text{CTDNA alone}}$ in °C. T_m measurements were performed in BPE buffer, in 1 cm quartz vials at 260 nm with a heating rate of 1 °C min^{-1}. The T_m values were obtained from first-derivative plots. [b] Drug concentrations that inhibit circulating HL60 leukemia cells from solid tumors growth by 50% after 72 h of incubation. Roscovitin and Rebeccamycin were used as internal standard for cell assays. [c] Drug concentrations that inhibit circulating adherent cells from solid tumors growth by 50% after 48 h of incubation. Roscovitin and Rebeccamycin were used as internal standard for cell assays.

on poly-acrylamide gels. The unwinding of DNA resulting from the insertion of the drug between base pairs is clearly evidenced with several compounds, in particular with **31** and **38**. In these cases, the topoisomer population is shifted to the top part of the gel at 20 μM (relaxation) and at higher concentrations, the DNA becomes positively supercoiled because of intercalation and it starts to migrate faster in the gel. Together with the CD data, this result effectively attests that these compounds intercalate into DNA.

The cytotoxicity was evaluated further using a pair of murine leukemia cells, either sensitive or resistant to the antitumor drug camptothecin (CPT) which is a reference topoisomerase I poison. The resistance of the P388CPT5 cells has been attributed to the expression of a deficient form of topoisomerase I as a result of a mutation in the *top1* gene of these cells.[30] The two mutations (Gly^{361}Val and Asp^{709}Tyr) in conserved regions of the *top1* gene strongly diminish the sensitivity of the cells to CPT, by a factor >100 (Table 3).

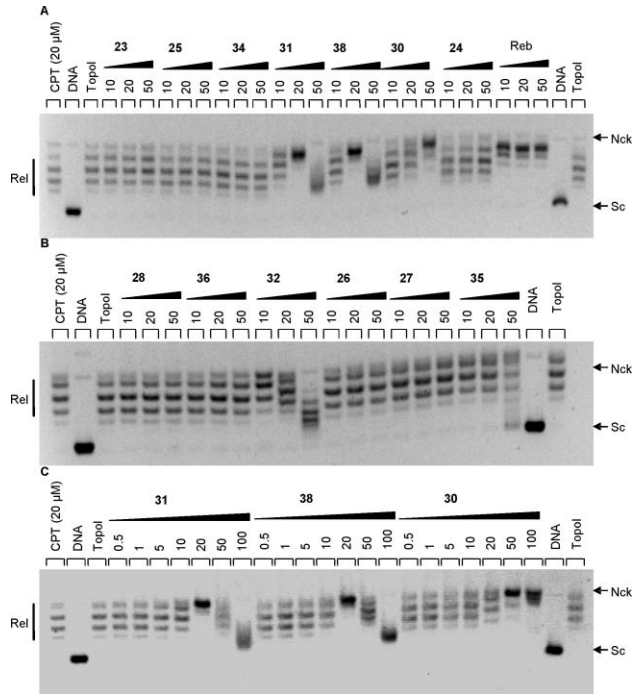


Fig. 4 Inhibition of topoisomerase I-mediated DNA plasmid pUC19 in the presence of graded concentrations of drugs. Plasmid DNA (120 ng, lane DNA) was incubated for 45 min at 37 °C with drug, then 30 min at 50 °C with 4 units of topoisomerase I (topogen Inc) in the absence (lane topoI) or in the presence of drug at the indicated concentrations (µM). Reaction was stopped with SDS and treatment with proteinase K. The DNA was analyzed by agarose gel electrophoresis. The gel was stained with ethidium bromide and photographed under UV light. Nck: nicked; Rel: relaxed; Sc: supercoiled.

In contrast, these mutations showed little effect on the cytotoxic potential of **31** and **32**, as is the case for rebeccamycin. Topoisomerase I is not the main target of these compounds. Nevertheless, the use of these additional pair of cell lines confirmed the cytotoxic bioactivity of **31–32**, with $IC_{50} < 1.0$ µM. Noteworthy, the higher cytotoxicity of **32** compared to **31** was observed in all cases, with

Table 3 Cytotoxicity on sensitive and resistant P388 cell line

Compound	P388 (IC_{50}/µM)	P388CPT5 (IC_{50}/µM)	RRI[a]
Camptothecin	0.031	3.310	107
Rebeccamycin	0.19	0.71	3.7
31	0.70	1.02	1.46
32	0.231	0.521	2.26

[a] Relative resistance index: IC_{50}(CPT-resistant)/IC_{50}(CPT-sensitive).

circulating HL-60 cells and P388 cells as well as with adherent cancer cells derived from solid tumors, such as human colon carcinoma Caco2 cells and human hepatocarcinoma Huh7 cells (Table 2). In addition, very actively proliferating transformed rat biliary epithelial cells were also found to be exquisitely sensitive to this molecule, with an IC_{50} of 700 nM (Table 2). Meanwhile, this compound was also found to be active onto non-tumoral poorly proliferating human skin fibroblastic cells, suggesting distinct signaling pathways that warrant further investigation.

In parallel, the effect on the cell cycle of P388 and P388CPT5 leukemia cells of DNA-binding compounds **30–32**, **38** was investigated (Fig. 5). Compounds **31** and **32** induced a strong accumulation of the cells in the G2 + M phase at 1 µM, as observed with camptothecin, whereas **38** and **30** showed no significant effect at the same concentration. A similar trend was observed with the topoisomerase I-mutated cell line P388CTP5. It is clear that the mutation of the *top1* gene does not impact on the activity of the two oxophenylarcyriaflavin compounds.

Kinase inhibition

It is well known that (hetaryl)pyrrolo[3,4-*c*]carbazole derivatives can induce selective protein kinase inhibition.[14–17,32] So we next tested the compounds for potential inhibition of CDK1, CDK5 and glycogen synthase kinase-3 (GSK3) to evaluate a possible selectivity (Table 4).[19] The non cytotoxic compound **36** showed a significant inhibitory activity (IC_{50} CDK1 = 1.3 µM, IC_{50} CDK5 = 0.9 µM IC_{50} GSK3 = 2.2 µM), without any selectivity, thus indicating the major role of the unsubstituted maleimide group in

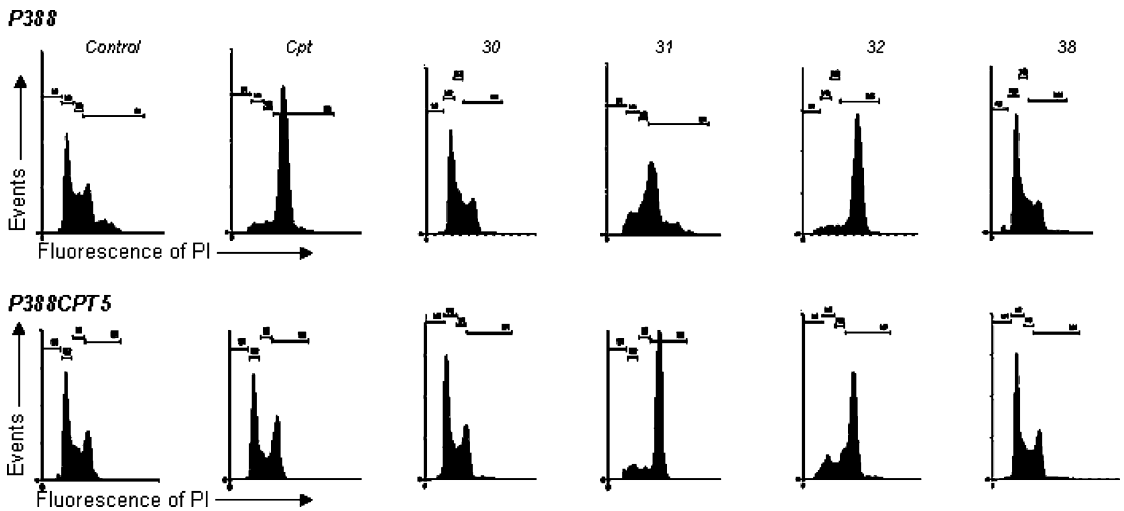
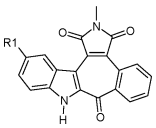
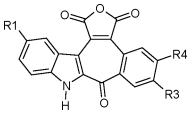
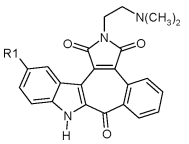
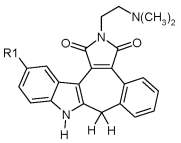
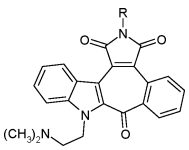
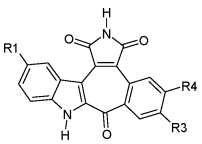


Fig. 5 Cell cycle distribution in CEM cells treated for 24 h with graded concentrations of **30–32**, **38**. Cells were analyzed with the FACScan flow cytometer. Data are the result of two independent experiments.

Table 4 Inhibition tests on CDK1, CDK5 and GSK3

Compound		$IC_{50}/\mu M$		
		CDK1	CDK5	GSK3
	**23** R1 = H	>10	ND	>10
	24 R1 = OH	75	10	>100
	**25** R1 = R3 = R4 = H	4.5	14	45
	26 R1 = H, R3 = R4 = OMe	>10	>10	>10
	27 R1 = H, R3 = R4 = OH	40	30	5.7
	28 R1 = OH, R3 = R4 = H	19	>100	>100
	**31** R1 = H	16	8.2	12
	32 R1 = OH	>10	>10	>10
	**30** R1 = H	4	9	20
	**38** R = $CH_2CH_2N(CH_3)_2$	2.6	4.8	10
	**34** R1 = R3 = R4 = H	4.3	3.9	18
	36 R1 = OH, R3 = R4 = H	**1.3**	**0.9**	**2.2**

this activity. The lack of the hydroxyl group impaired the inhibition indicating that the indolic hydroxyl group of **36** gives a favourable interaction with the enzyme catalytic site (compound **34**, IC_{50} CDK1 = 4.3 μM, IC_{50} CDK5 = 3.9 μM IC_{50}, GSK3 = 18 μM). The selectivity of CDKs *versus* GSK3 was enhanced when the indolic hydroxy group was absent. Surprisingly the last unsubstituted maleimide compound **35**, which is hydroxylated on the phenyl ring, was inactive against all the tested kinases but exhibited strong cytotoxic activities against Huh7 and F1 cell lines. This result indicates also that kinase inhibition and cytotoxicity were not correlated. Compound **36** constitutes an interesting scaffold from which more potent and more selective inhibitors could potentially be designed.

Conclusion

To sum up, in this paper we designed new polysubstituted oxophenylarcyriaflavins starting from indoles using efficient syntheses. Preparation of these compounds was achieved using a Suzuki or a Stille procedure whereas formation of the central tropone ring required an intramolecular electrophilic reaction. Different substitutions either on the indolic nucleus, on the phenyl ring or on the maleimide moiety were successfully achieved to provide access to a new potential anticancer agent family. Some molecules proved to be potent cytotoxic agents able to interfere with the cell cycle of cancer cells. DNA binding, but not topoisomerase inhibition, seems to play a role in the cytotoxic action. CDK1, CDK5 and GSK3 are apparently not major targets for these compounds but the oxophenylarcyriaflavin scaffold may be further exploited to generate kinase inhibitors.

Experimental section

A. Chemistry

^1H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX 250 instrument using $CDCl_3$ or DMSO-d_6. The chemical shifts are reported in ppm (δ scale) and all J values are in Hz. The following abbreviations are used: singlet (s), doublet (d), doubled doublet (dd), triplet (t), multiplet (m), quaternary carbon (Cq). Melting points are uncorrected. IR absorptions were recorded on a Perkin Elmer PARAGON 1000 PC and values were reported in cm^{-1}. MS spectra (Ion Spray) were performed on a Perkin Elmer Sciex API 300 or on Avatar 320 equipped using an ATR (Ge) technique.

HRMS were performed by the Centre Régional de Mesures Physiques de l'Ouest (CRMPO, Rennes) on a high resolution mass spectrometer with double focalisation Varian Mat 311 using electronic impact. Monitoring of the reactions was performed using silica gel TLC plates (silica Merck 60 F_{254}). Spots were visualized by UV light at 254 and 356 nm. Flash chromatography columns were performed using silica gel 60 (0.040–0.063 mm, Merck). Microwave experiments were performed on a Biotage Initiator apparatus. For compounds **4**, **12–22** in the ^{1}H NMR data the H′ refers to phenyl ring protons. Synthesis and experimental data of compounds **3**, **15**, **23**, **25**, **29–31**, **34**, **37**, **38** were previously reported.[22]

For the preparation and characterization of compounds **4**, **10–14**, **16–22**, **24**, **26–28**, **32**, **33**, **35**, **36**, see the ESI.

B. Biological studies

Topoisomerase inhibition assays. These were performed as previously described.[31]

DNA binding measurements

Melting curves were measured with an Uvikon 943 spectrophotometer coupled to a Nesab RTE111 cryostat. Titrations of the drug with DNA, covering a large range of drug/DNA-phosphate ratios (D/P), were performed by adding aliquots of a concentrated drug solution to a constant DNA solution (20 μM). T_m measurements were performed in BPE buffer pH 7.1 (6 mM Na2HPO4, 2 mM NaH_2PO_4, 1 mM EDTA). The temperature inside the cuvette (10 mm path length) was increased over the range 20–100 °C with a heating rate of 1 °C min^{-1}. The "melting" temperature T_m was taken as the mid-point of the hyperchromic transition.

Cell culture and survival assay

Human CEM and CEMC2 leukemia cells were obtained from the American Tissue Culture Collection. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 4.5 g L^{-1} glucose, 10 mM HEPES, 1 mM sodium pyruvate, penicillin (100 IU mL^{-1}), and streptomycin (100% g mL^{-1}). The cytotoxicity on these cells of the tested compounds was assessed using a cell proliferation assay developed by Promega (CellTiter 96 Aqueous one solution cell proliferation assay). Briefly, 2×10^4 exponentially growing cells were seeded in 96-well microculture plates with graded drug concentrations in a volume of 100 μL. After 72 h incubation at 37 °C, 20 μL of the tetrazolium dye was added to each well, and the samples were incubated for a further 2 h at 37 °C. Plates were analyzed on a Labsystems Multiskan MS (type 352) reader at 492 nm.

Skin diploïd fibroblastic cells were provided by BIOPREDIC International Company (Rennes, France). Caco-2 cells and Huh7 cells were obtained from the ECAC collection. Cells were grown according to ECAC recommendations. RLEC-F1 clone is derived from an established rat biliary epithelial cell line as previously described.[28] The toxicity test of the compounds on these cells was as follows: 4×10^3 cells were seeded in 96 multiwell plates and left for 24 h for attachment, spreading and growing. Then, they were exposed for 48 h to increasing concentrations of the compounds, ranging from 0.1 to 25 μM in a final volume of 80 μl of culture medium. They were fixed with 4% paraformaldehyde solution and nuclei were stained with Hoechst 3342 and counted using automated imaging analysis (Simple PCI software).

Cell cycle analysis

For flow cytometric analysis of DNA content, 0.7×10^6 cells in exponential growth were treated with graded concentrations of the tested drug for 24 h and then washed with 1 mL of PBS. After centrifugation, the cell pellet was resuspended in 1 mL of cold ethanol for 24 h at −0 °C. The ethanol was removed, and the pellet was washed with 1 mL PBS and then incubated for 30 min in a solution containing 50 μg mL^{-1} PI and 100 μg mL^{-1} RNase. Samples were analyzed on a Becton Dickinson FACScan flow cytometer using CellQuest software, which was also used to determine the percent of cells in the different phases of the cell cycle. PI was excited at 488 nm, and fluorescence analyzed at 620 nm on channel Fl-2.

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