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Cytotoxicity mechanisms of pyrazino[1,2-*b*]isoquinoline-4-ones and SAR studies

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ABSTRACT

The cytotoxicity showed by **1b**, an interesting representant of the title compounds, for HT-29 human colon cancer cells (CI_{50} value of 1.95×10^{-7} M) has been related to the induced cell death at the G2 phase and not to DNA damage. This compound promotes the degradation of components of the G2/M checkpoint machinery, in particular cdc2, Cyclin B1 and Wee1, which represents a novel mechanism of cytotoxicity. Degradation of Wee1 seems to be mediated by proteasome activity but degradation of cdc2 has to occur through a different mechanism. The activity of **1b** on G2 cell cycle components suggests that tumor cells that are arrested in G2/M by anticancer drugs like cisplatin could be targeted by compound **1b**, increasing the apoptosis induction, and that their optimized analogs might be useful in the treatment of colon cancer through combination therapies with cisplatin or other anticancer drugs that affect the cytoskeleton integrity such as taxol and taxotere.
SAR studies with compounds obtained by manipulation of the N(2) and C(4)-functional groups and the C(6)-chain of compound **1b** have confirmed the importance of these structural features in the in vitro antitumor activity. Fused oxazolidine derivatives as compound **5** were inactive, and the lack of activity found in the replacement of the C(4)-lactam by a cyanoamine function, as in compounds **8–10**, could be explained considering that their all-*syn* relative configuration makes them too stable to generate alkylating iminium species.

1. Introduction

A synthetic strategy developed in our group to obtain analogues of tetrahydroisoquinoline natural products as potential antitumor compounds, gives rise first to pyrazino[1,2-*b*]isoquinolines, which are subsequently converted into pentacyclic or octacyclic derivatives.[1] This approach allows the biological evaluation of simplified structures in order to establish pharmacophoric features. In this context, we have recently reported that some tricyclic compounds retain significant in vitro cytotoxicity, in spite of their structural simplicity and the lack of the characteristic N(2)–C(21) hemiaminal or aminonitrile motifs that have been presumed to be essential to cytotoxic activity mediated by the generation of DNA-alkylating electrophilic C(21)-iminium ions.[2] Initial studies to investigate the cytotoxicity mechanisms of these simplified structures performed with compound **1a** (Scheme 1), with a CI_{50} value for A-549 cells of 5.68×10^{-6} M, showed that it does not produce DNA damage, but induces apoptosis triggered directly from the G2/M phase through a process that is not mediated by activation of pro-apoptotic kinases JNK and P38, or by activation of AKT.[3] In this paper we report the so far obtained data about the biological mechanism of compound **1b**, which is selectively active against the colon cancer cell line HT-29 with a CI_{50} value of 1.95×10^{-7} M. We also report the synthesis and in vitro cytotoxicity of some **1b** derivatives, obtained by manipulation of the C(6)-side chain and reduction of C(4)-lactam and the C(2)-carbamate functions, in order to establish some structure–activity relationships.

2. Results and discussion

2.1. Chemistry

Treatment of compound **2**[3] with an excess of $LiAl(OEt)_2H_2$ at room temperature gave a mixture of the *N*-methyl-piperazine **3** and the rather unstable *N*-methyl-oxazolidine **4**. Taking into account that a carbamate function is less reactive than a lactam function,[4] the chemoselective reduction of the C(4) lactam group was achieved with two equivalents of $LiAlH_4$ at low temperature, to give quantitatively the *N*-alkoxycarbonyl-oxazolidine **5**. Attempting the isolation of a C(4)-hydroxy hemiaminal compound, the OH group of **2** was protected by treatment with *tert*butyl(diphe-

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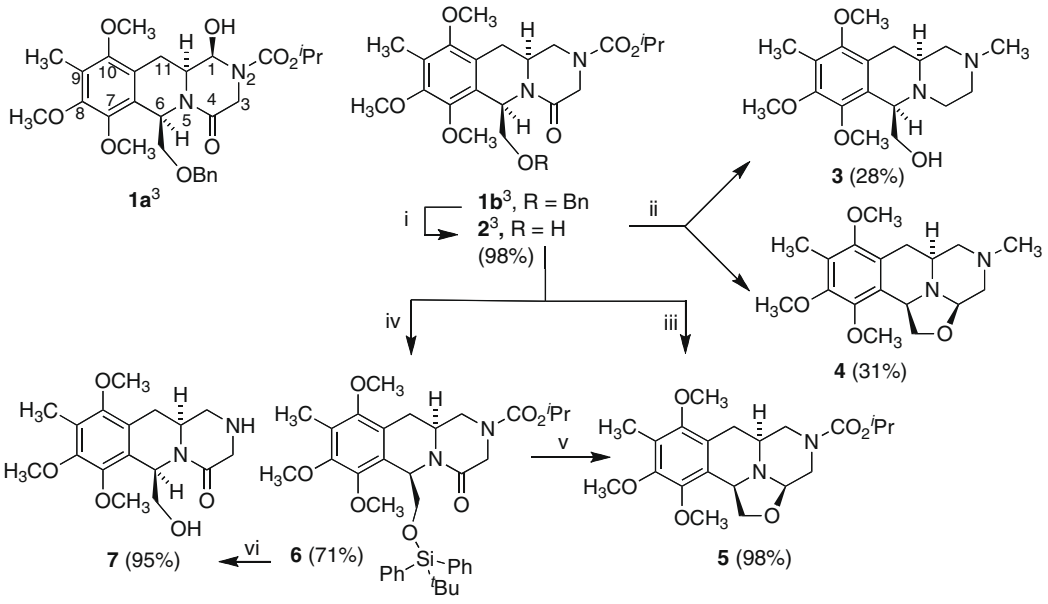
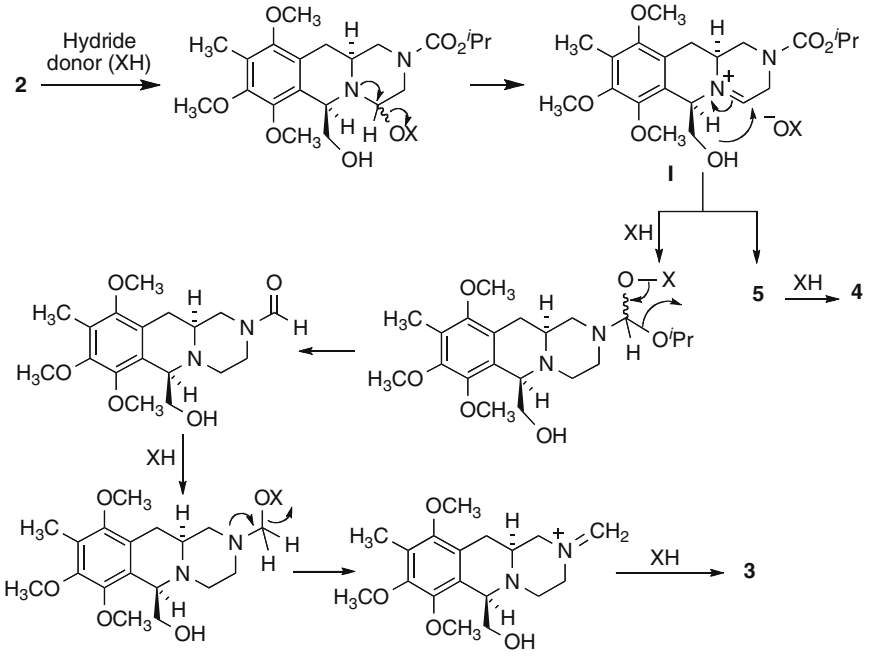


Scheme 1. Reagents and conditions: (i) H_2 (3.5 atm.), 10% C–Pd, EtOH, 70 °C, 16 h; (ii) $LiAl(OEt)_2H_2$ (20 equiv), THF, rt, 35 min; (iii) $LiAlH_4$ (2 equiv), THF, −17 °C, 30 min., then 0 °C, 1 h; (iv) TBDPSCl (5 equiv), DMAP (10 equiv), DCM, rt, 3.5 h; (v) $LiAlH_4$ (1.2 equiv), THF, −17 °C, 30 min, then 0 °C, 1 h; (vi) TFA/H_2SO_4 (20:1), rt, 16 h.

nyl)silyl chloride to give **6**, but its reduction also afforded compound **5**. Deprotection of the NH and OH groups of compound **6** in acid conditions gave **7** (Scheme 1).

The putative iminium intermediate **I** produced in the partial reduction of the C(4)-carbonyl group of **2**, gave compound **3** by further reduction. Alternatively, it cyclized to **5** by an intramolecular addition of the hydroxy group, giving **4** by subsequent reduction of the carbamate function (Scheme 2). Similar ring closure reactions involving iminium cations to get fused oxazolidine tetrahydroisoquinoline compounds have been previously reported,[5–9] but the transformation of **2** into **5** represents the highest yielding process. The cascade of reduction reactions affecting to lactam and carbamate functions in the one-pot transformation of **2** into **3** is also interesting, because reductions of *N*-alkyl or *N*-arylcarbamates to *N*-methylalkyl or *N*-methylarylamines are too slow and have been scarcely used in synthesis.[10] The transformation of **6** into **5** probably involves the intramolecular transfer of the TBDPS group to the 4-hydroxy intermediate with subsequent generation of **I**.

Compounds **4** and **5** contain four of the rings present in several antitumor natural products such as quinocarmycin, bioxolamycin α_2, and tetrazomine (Fig. 1),[11] being known that the hemiaminal portion of these oxazolidine systems makes them rather unstable and very reactive against nucleophiles, including water.[12,13] In fact, compounds **4** and **5** were purified and characterized after a fast column chromatography, but had to be stored under an argon atmosphere at 10 °C.



Scheme 2.

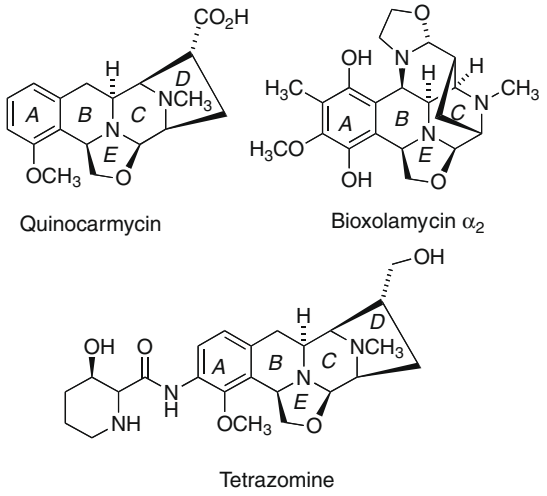
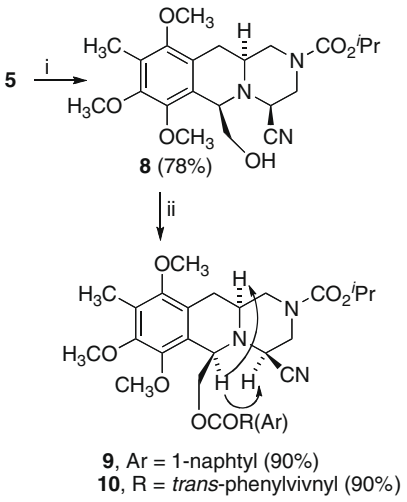


Figure 1.

As we have already mentioned, a cyanopiperazine core or a hemiaminal equivalent function is considered essential in most antitumor natural products belonging to the tetrahydroisoquinoline family for effective DNA alkylation at the minor groove through the generation of intermediate iminium species.[2,14,15] In order to get these structural motifs, compound **5** was treated with a mixture of trimethylsilyl cyanide and boron trifluoride as a Lewis acid.[16–18] This reaction gave the cyanopiperazine **8** as a single diastereoisomer in a very good yield. The overlapping of H(4) and H(11)-signals in this compound prevented the assignment of the C(4), C(6) and C(11a) relative stereochemistry by ^{1}H NMR/NOE experiments, but this study was possible in the *O*-acyl derivatives **9** and **10**. These experiments showed unequivocally that protons H-4, H-6 and H-11a have a *syn*-relationship (Scheme 3).

The relative stereochemistry of cyano derivatives **8–10**, where the cyano group is *syn* respect to the C(6)-chain, differs from that found in natural alkaloids such as saframycin A,[19,20] and in compounds obtained by reductive cyanation of a lactam function in 6,15-iminoisoquino[3,2-*b*]-3-benzazocines such as cribrostatin 4 derivatives[21] and phthalascidin.[22] A similar ring-opening of a oxazolidine ring in quinocarmycin gave the promising anticancer drug CD-521, with a cyano group *anti* respect to the side-chain[23] (Fig. 2).



Scheme 3. Reagents and conditions: (i) TMSCN (2.7 equiv), $BF_3{\cdot}OEt_2$ (0.7 equiv), −30 °C, 1.5 h; (ii) R(Ar)CO_2H, EDC (2 equiv), DMAP (1.1 equiv), DCM, rt, 21 h.

The cyano group is located in all these compounds at the less-hindered α-face, although Williams and co-workers have reported a similar all-*syn* relative stereochemistry in the ring-opening cyanation of a oxazolidine precursor related to compound **8**.[7]

It appears that the presence of the 1,3-bridge in the pyrazino[1,2-*b*]isoquinoline skeleton, which is present in the above mentioned 6,15-iminoisoquino[3,2-*b*]-3-benzazocines, prevents the cyanide attack to the β-face of the iminium ion generated by partial reduction/dehydration of a C(4)-lactam or by ring-opening of a 4,6-epoxymethane bridge. In our case, the incipient iminium species **III** generated from the ion-pair **II**, could be attacked by the cyanide anion *anti*-respect to the hydroxymethyl group, but the putative α-amino nitrile **A** thus formed has an axial cyano group which is *trans*-antiperiplanar to the unshared electron-pair of the bridge-head nitrogen atom, and the cyanide anion would be readily expelled regenerating **III**. The *syn*-attack of this anion would give **8**, the product of thermodynamic control that may exhibit a reduced capacity for forming covalent bonds to DNA[24] (Scheme 4).

3. Pharmacological results

3.1. Antiproliferative activity of compounds 2–10

In contrast to the high selective cytotoxicity found in compound **1b**,[3] compounds **2**–**10** were inactive at concentrations <100 mM when tested against representative human tumor cell lines MDA-MB-231, A-549, and HT-29.

3.2. Effects on cell cycle transitions of compound 1b

Flow cytometric analysis was used to evaluate the effects of compound **1b** on cell cycle progression of HT-29 cells by measuring their DNA content, which is proportional to the level of fluorescence.[25] We choose the dose of 41 μg/mL because it induced a significant reduction on cell viability (80%), and cisplatin (CDDP, 5 μg/mL) as a control because it induces apoptosis in tumor cells that is triggered when the cells are in G1. Effectively, cisplatin induced apoptosis, measured as the sub-G0 population and a reduction in the number of HT-29 cells in G1, suggesting that apoptosis was triggered from this phase of cell cycle. Compound **1b** also induced apoptosis, but it did not significantly changed the number of cells in G1 or G2 phases, although slightly decreased the number of cells in the S phase. These data suggested that **1b**, the same as the previously studied compound **1a**,[3] affects the G2/M checkpoint of the cell cycle.

In order to verify in which phase of cell cycle were the cells driven to apoptosis, we synchronized the cells in G2 by treating them with the microtubule agent nocodazole.[26] In this situation 89% of the cells were found in G2 as expected (Table 1). When these cells were subsequently treated with cisplatin, we observed a significant reduction in the proportion of cells that enter again in G1 (72 h after treatment only a 2.5-fold increase), but when these cells were treated with compound **1b**, they enter again efficiently in G1 (14-fold increase). However, treatment with cisplatin and later with nocodazole slightly decreased the number of cells in G2 (1.6-fold), while an important decrease was observed for compound **1b** (fivefold). Altogether, these data indicate that cisplatin was inducing an exit of cells mainly from G1 and compound **1b** from G2.

3.3. Alteration of signaling pathways related to DNA damage and apoptosis induction

Since treatment with compound **1b** was inducing apoptosis and cell cycle alterations, we checked if DNA damage signaling pathways were activated in response to treatment with this

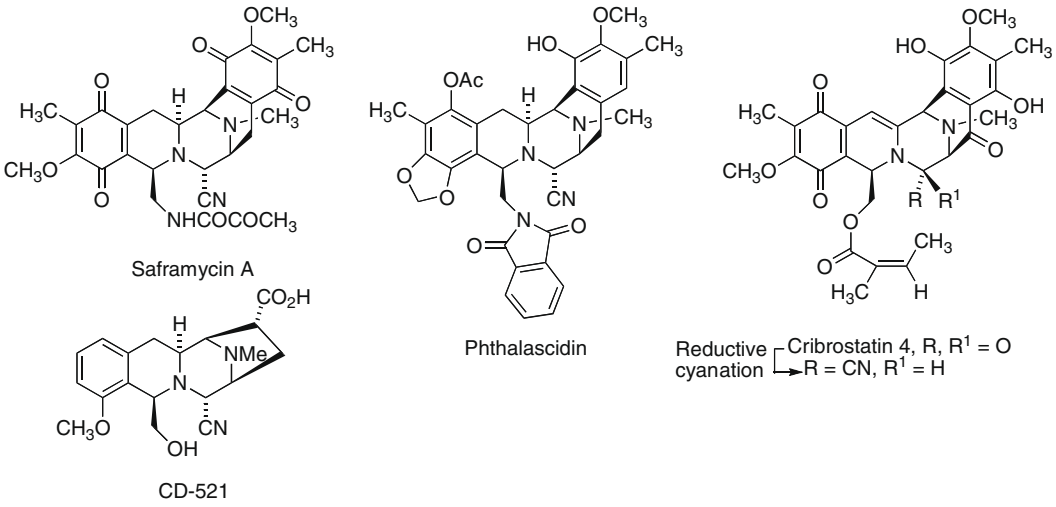
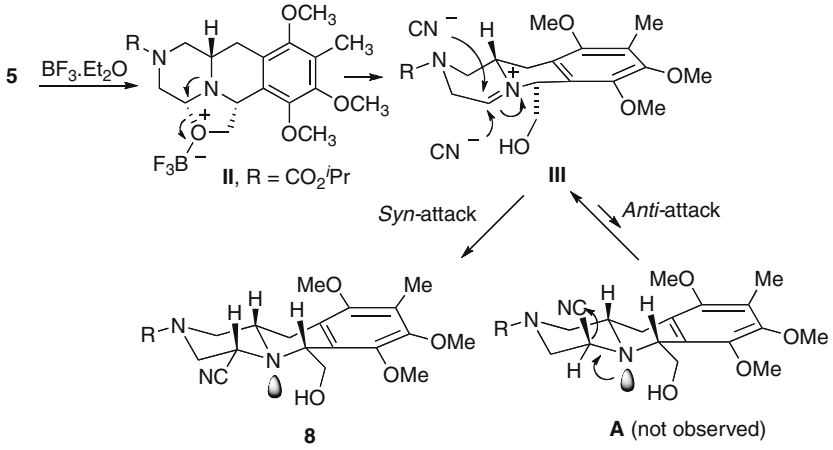


Figure 2.



Scheme 4.

Table 1
Cell cycle distribution[a] by flow cytometry in HT-29 cells treated for 72 h after nocodazole pretreatment

Compound	Apoptosis (%)		G1 (%)		S phase (%)		G2/M (%)	
	−Noc	+Noc	−Noc	+Noc	−Noc	+Noc	−Noc	+Noc
Control	2.4	1.6	48.1	2.2	22.7	5.9	26.4	89.2
Cisplatin	40.0	28.2	12.8	5.5	13.8	9.9	31.9	54.4
1b	29.9	35.6	45.9	31.0	12.8	13.4	10.4	18.3

HT29 cells pretreatment with 0.2 μg/mL nocodazole overnight (+Noc) and without pretreatment (−Noc) were stimulated during 72 h with 41 μg/mL of compound **1b** or 5 μg/mL of CDDP, respectively. After, cells were fixed and analyzed by flow cytometry as indicated.

[a] Percentage of cells in each phase of the cell cycle as measured by flow cytometry.

compound. As a positive control, we treat cells with the DNA damage agent cisplatin and studied phosphorylation of p53 on Ser15 as well as phosphorylation in different residues of the cell cycle kinases Chk1 and Chk2 that are usually phosphorylated in response to DNA damage. As previously described,[27] cisplatin induced phosphorylation of p53, of Chk2 on residues Ser19, Thr68, and Chk1 on Ser345, while compound **1b** did not induce phosphorylation of p53, Chk1 or Chk2 even after 24 h of treatment (Fig. 3).

We also studied if treatment with compound **1b** was able to induce an increase in histone γ-H2A·X using cisplatin as a positive control. Results again demonstrated that while cisplatin induced a time dependent activation of histone γ-H2A·X, compound **1b** did not, even after longer times of incubation (Fig. 4).

Since pretreatment with compound **1b** followed by nocodazole induced a marked decrease in the proportion of cells in G2, we studied if **1b** was affecting the activation of the Cyclin-dependent kinase Cdk1 (also known as cell division cycle 2 or cdc2), a member of the Ser/Thr protein kinase family encoded by the gene $p34^{cdk1}$ which, when bound to Cyclin B1, allows a dividing mammalian cell to enter into mitosis from G2. We observed that, while treatment with cisplatin induced phosphorylation of cdc2 and of its inhibitory protein Wee1, suggesting an activation of the G2/M checkpoint, compound **1b** seemed to induce dephosphorylation of cdc2 and Wee1. We determined that this was due in fact, to a decrease in the levels of both proteins (Fig. 5). Since cdc2 has to be phosphorylated at the end of the cycle to induce its arrest, if it is permanently dephosphorylated or absent as we have seen to occur in HT-29 cells after treatment with **1b**, cells enter into apoptosis.

In order to verify if compound **1b** was effectively inducing a decrease of cdc2 and Wee1 levels, we pre-treated cells with nocodazole,[26] which induces an accumulation of cdc2. When this experiment was done with cisplatin, we did not observe any change in phosphorylated cdc2 or cdc2 protein levels (Fig. 6). In the control, an inhibition of cdc2 and induction of cdc2 degradation

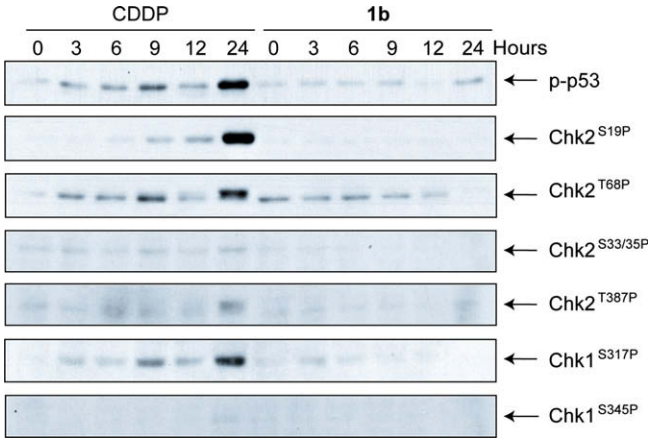


Figure 3. Effect of CDDP and **1b** in phosphorylation of different residues of p53 and cell cycle kinases Chk1 and Chk2. HT29 cells were treated with 41 μg/mL of compound **1b** or 5 μg/mL of CDDP, respectively, and harvested at the times indicated. After, cells were lysed and analyzed by western blot with antibodies against $p53^{S15P}$, and phosphorylation in different residues of Chk1 and Chk2.

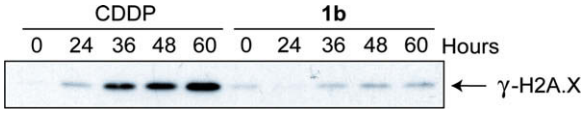


Figure 4. Histone γ-H2A·X increased after treatment with cisplatin but not after treatment with **1b**. HT29 cells were treated with 5 μg/mL of CDDP or 40 μg/mL of compound **1b**, respectively, and harvested at the times indicated. After, cells were lysed and analyzed by western blot with antibody against γ-H2A·X.

was observed after treatment with 10% FBS, as a consequence of progress through cell cycle. On the other hand, compound **1b** induced a decrease in levels of phosphorylated cdc2 and cdc2, which is accelerated when cells are pretreated with nocodazole. Altogether, these results indicate that compound **1b**, should be inducing degradation of both cdc2 and Wee1, and this may be contributing to apoptosis induction.

As compound **1b** seemed to induce degradation of cdc2 and Wee1, we tried to test the involvement of proteasome by co-treating cells with compound **1b** and MG-132, a potent proteasome

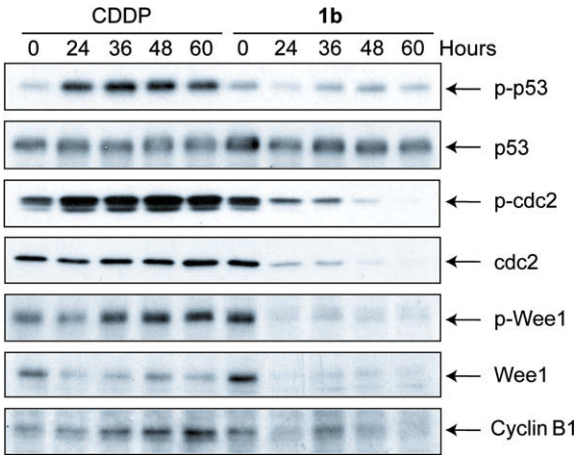


Figure 5. Different activation of p53, cdc2, Wee1 and Cyclin B1 after treatment with cisplatin and compound **1b**. HT29 cells were treated with 5 μg/mL of CDDP or 41 μg/mL of compound **1b**, respectively, and harvested at the times indicated. After, cells were lysed and analyzed by western blot with antibodies against $p53^{S15P}$, p53, p-cdc2, cdc2, p-Wee1, Wee1 and Cyclin B1.

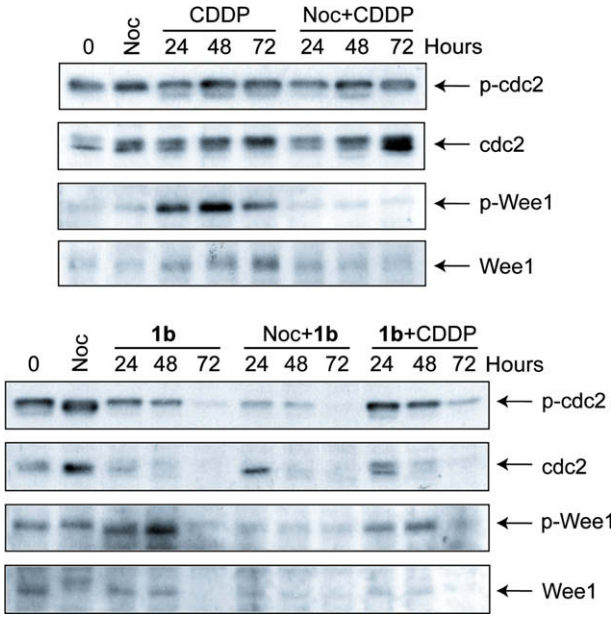


Figure 6. Treatment with compound **1b** accelerates degradation of cdc2 and p-Wee1 after nocodazole treatment. HT29 cells pretreatment with 0.2 μg/mL of nocodazole overnight and HT29 without pretreatment were stimulated with 5 μg/mL of CDDP (first panel) or 41 μg/mL of compound **1b** (second panel), or the combination of both. After, cells were harvested at the times indicated, lysed and analyzed by western blot with antibodies against p-cdc2, cdc2, p-Wee1 and Wee1.

inhibitor[28] (Fig. 7). This experiment revealed that the decrease of Wee-1 was partially prevented by MG-132 but degradation of cdc2 was not. This suggests that compound **1b** may promote the proteasome-mediated degradation of Wee-1 through activation of the anaphase-promoting complex (APC or cyclosome). On the other hand, MG-132 should be inhibiting degradation of a protease that acts on cdc2, since the treatment of cells with this proteasome inhibitor accelerated the kinetic of cdc2 degradation.

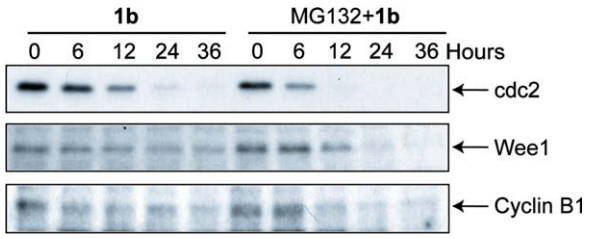


Figure 7. Treatment with compound **1b** accelerated degradation of cdc2 but no Wee1 and Cyclin B1 after proteasome inhibitor treatment. HT29 cells pretreatment with 10 μM MG132 for 30 min and without pretreatment were stimulated with 41 μg/mL of compound **1b**. After, cells were harvested at the times indicated, lysed and analyzed by western blot with antibodies against cdc2, Wee1 and Cyclin B1.

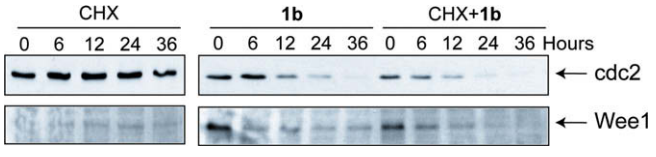


Figure 8. Treatment with compound **1b** induces degradation of cdc2 and Wee1 after inhibition of protein synthesis with CHX. HT29 cells pretreatment with 300 μM cycloheximide (CHX) for 1 h and HT29 without pretreatment were stimulated with 41 μg/mL of compound **1b**. After, cells were harvested at the times indicated, lysed and analyzed by western blot with antibodies against cdc2 and Wee1.

Table 2
Cell cycle distribution by flow cytometry in HT-29 cells treated for 24 h

Compound	Apoptosis (%)	G1 (%)	S phase (%)	G2/M (%)
Control	2.4	48.1	22.7	26.4
Cisplatin	2.7	27.5	49.9	18.7
1b	4.3	61.4	15.2	16.4
1b + cisplatin	20.5	55.1	13.4	9.4

HT29 cells were stimulated for 24 h with 41 μg/L of compound **1b**, 5 μg/mL of CDDP, or both. After, cells were fixed and analyzed by flow cytometry as indicated.

In order to verify that compound **1b** promotes the degradation of cdc2 through other mechanisms, HT-29 cells were pre-incubated with cycloheximide, an inhibitor of protein biosynthesis that acts specifically on the 60S subunit of eukaryotic ribosomes[29] (Fig. 8). Cdc2 protein levels were decreased with a faster kinetic when cells were preincubated with cycloheximide, indicating that indeed, compound **1b** was inducing the degradation of both cdc2 and Wee1.

The activity of compound **1b** on G2 cell cycle components suggests that tumor cells that are arrested in G2/M by anticancer drugs like cisplatin could be targeted by compound **1b**. Indeed, when we incubated cells with compound **1b** and cisplatin, we observed an increase in apoptosis induction (Table 2).

4. Conclusions

The low cytotoxicity found in the compounds **2–10** here described indicates that the cytotoxicity of **1b** to HT-29 human cancer cells has to be related to structural features additional to the C(4)-lactam group especially the benzyloxymethyl C(6)-substituent. Compounds **2**, **3**, **7** and **8** with a C(6)-hydroxymethyl group were inactive. Previous studies with lactam containing compounds in these series of our group[1] and others,[24] have shown that they are capable of exerting cytotoxic effects through mechanisms alternative to DNA alkylation. In contrast to the substantial cytotoxicity increase found in cribrostatin 4 after the replacement of a similar lactam for a cyanoamine function,[21] compounds **8–10** were also inactive. It is possible that their all-*syn* relative configuration makes them too stable to generate alkylating iminium species.

The **1b** induced cell death at the G2 phase in HT-29 cell represents a novel mechanism of cytotoxicity, since it does so by inducing degradation of components of the G2/M checkpoint machinery. It induced the degradation of cdc2 and Cyclin B1, proteins that drive cells through the G2 phase of cell cycle to mitosis, and it also induces degradation of Wee1, probably as a consequence of the degradation of cdc2 since it seems to involve a degradation mechanism (proteasome dependent) different to that induced to degrade cdc2.

When we studied the effect of the combined treatment of cisplatin and compound **1b** on cdc2 protein levels, we found an increase in cdc2 degradation (Fig. 6) similar to that observed by treatment with compound **1b**. Finally, the combination of compound **1b** and cisplatin induces an important increase in apoptosis (Table 2). In other words, these data suggest that pyrazino[1,2-*b*]isoquinoline-4-ones might be useful in the treatment of colon cancer through combination therapies with anticancer drugs like cisplatin. These observations would be valid also for compounds that affect the cytoskeleton integrity, such as taxol and taxotere.

5. Experimental section

5.1. General experimental information

All reagents were of commercial quality and were used as received. Solvents were dried and purified using standard techniques. Reactions were monitored by thin layer chromatography, on aluminum plates coated with silica gel with fluorescent indicator. Separations by column flash chromatography were performed on silica gel with 40–63 mm particle size. Melting points were measured in a hot stage microscope, and are uncorrected. Infrared spectra were recorded on a FT-IR spectrophotometer, with solid compounds compressed into KBr pellets and liquid compounds examined as films on NaCl disks. NMR spectra were obtained at 250 MHz for ^{1}H and 63 MHz for ^{13}C, with $CDCl_3$ as solvent (Servicio de Resonancia Magnética Nuclear, Universidad Complutense). Elemental analyses were determined by the Servicio de Microanálisis Elemental, Universidad Complutense.

5.2. General procedure to obtain compounds 3 and 4

$LiAlH_2(OEt)_2$ was prepared by the addition of dry ethyl acetate (1.6 mL, 16.2 mmol) to a 2 M suspension of lithium aluminum hydride in dry THF (81 mL, 28 mmol) under argon atmosphere, cooled in ice water, and stirring at 0 °C for 2 h. Then, compound **2** (340 mg, 0.81 mmol) in dry THF (6 mL) was added dropwise, and was additionally stirred for 35 min at room temperature. The reaction mixture was quenched by addition of ice and extracted with ethyl acetate (20 mL × 3). The extracts were washed with H_2O and with a saturated aqueous solution of NaCl, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo.

5.2.1. (6*R**,11a*S**)-6-Hydroxymethyl-7,8,10-trimethoxy-2,9-dimethyl-1,2,3,4,11,11a-hexahydro-6*H*-pyrazino[1,2-*b*]isoquinoline (3)

The residue was purified by flash column chromatography (3:7 EtOAc/methanol) to give **3** (90 mg, 28%) as an orange solid: mp 45–47 °C; IR (film) *ν*, 3398, 1462 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$) *δ* 3.86 (s, 3H), 3.80 (s, 3H), 3.72 (s, 3H), 3.67 (m, 2H), 3.42 (m, 1H), 3.19 (m, 2H), 2.91 (m, 1H), 2.86 (m, 2H), 2.58 (m, 2H), 2.37 (m, 1H), 2.27 (m, 1H), 2.31 (s, 3H), 2.20 (s, 3H); ^{13}C NMR (63 MHz, $CDCl_3$) *δ* 152.2 (C), 149.3 (C), 146.7 (C), 124.4 (C), 123.9 (C), 123.5 (C), 61.2 (CH_2), 61.1 (CH), 60.4 (CH_3), 59.9 (CH_3), 59.8 (CH_3), 59.2 (CH_2), 55.7 (CH_2), 47.6 (CH_2), 46.4 (CH_3), 45.7 (CH), 20.1 (CH_2), and 9.3 (CH_3). Anal. Calcd for $C_{18}H_{28}N_2O_4$: C, 64.26; H, 8.39; N, 8.33. Found: C, 64.12; H, 8.24; N, 8.17.

5.2.2. (4*R**,6*R**,11a*S**)-7,8,10-Trimethoxy-2,9-dimethyl-4,6-oxymethylene-1,2,3,4,11,11a-hexahydro-6*H*-pyrazino[1,2-*b*]isoquinoline (4)

The residue was purified by flash column chromatography (7:3 EtOAc/methanol) to give **4** (10 mg, 31%) as an orange solid: mp 73–75 °C; IR (film) *ν*, 2939, 1668 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$) *δ* 4.70 (s, 1H), 4.50 (t, *J* = 8.5 Hz, 1H), 4.27 (t, *J* = 8.5 Hz, 1H), 3.79 (s, 3H), 3.77 (s, 3H), 3.68 (s, 3H), 3.62 (m, 1H), 3.30 (d, *J* = 12.8 Hz, 1H), 2.88 (m, 1H), 2.87 (br s, 1H), 2.82 (d, *J* = 12.8 Hz, 1H), 2.57 (dd, *J* = 12.8 and 2.5 Hz, 1H), 2.45 (dd, *J* = 15.8 and 12.0 Hz, 1H), 2.36 (s, 3H), 2.22 (s, 3H), 2.11 (t, *J* = 10.8 Hz, 1H); ^{13}C NMR (63 MHz, $CDCl_3$) *δ* 151.9 (C), 149.5 (C), 146.5 (C), 126.3 (C), 123.8 (C), 123.5 (C), 89.8 (CH), 68.5 (CH_2), 60.0 (CH_3), 59.9 (CH_3), 59.8 (CH_3), 59.6 (CH), 59.0 (CH_2), 55.1 (CH_2), 49.0 (CH), 45.8 (CH_3), 27.7 (CH_2), and 9.2 (CH_3). Anal. Calcd for $C_{18}H_{26}N_2O_4$: C, 64.65; H, 7.84; N, 8.38. Found: C, 64.32; H, 7.59; N, 8.06.

5.2.3. (4*R**,6*R**,11a*S**)-2-Isopropyloxycarbonyl-7,8,10-trimethoxy-9-methyl-4,6-oxymethylene-1,2,3,4,11,11a-hexahydro-6*H*-pyrazi no[1,2-*b*]isoquinoline (5)

To a stirred solution of compound **2** (257 mg, 0.61 mmol) in dry THF (61 mL) under argon atmosphere and cooled at −17 °C, was added a 2 M suspension of lithium aluminum hydride in dry THF (0.61 mL, 1.22 mmol). After stirring at −17 °C for 30 min and 1 h at 0 °C, the reaction mixture was quenched by addition of ice and extracted with ethyl acetate (20 mL × 3). The extracts were washed with H_2O and with a saturated aqueous solution of NaCl, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo.

Due to the instability of compound **5**, we report the spectroscopic data of the crude product; IR (film) ν, 2940, 1698 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ 4.95 (sept, J = 6.2 Hz, 1H), 4.60 (br s, 1H), 4.48 (m, 2H), 4.24 (m, 2H), 3.84 (s, 3H), 3.81 (s, 3H), 3.70 (s, 3H), 3.60 (dd, J = 8.8 and 7.4 Hz, 1H), 3.41 (m, 1H), 2.81 (m, 3H), 2.42 (dd, J = 15.1 and 10.4 Hz, 1H), 2.21 (s, 3H), 1.31 (d, J = 6.2 Hz, 6H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 155.6 (C), 151.9 (C), 149.5 (C), 146.4 (C), 125.9 (C), 123.0 (C), 122.9 (C), 88.6 (CH), 69.0 (CH), 68.4 (CH_2), 60.0 (CH_3), 59.9 (CH_3), 59.9 (CH), 59.6 (CH_3), 48.2 (CH), 46.8 (CH_2), 43.6 (CH_2), 27.1 (CH_2), 22.2 (CH_3), and 9.2 (CH_3).

5.2.4. (6*R**,11a*S**)-4-*tert*Butyldiphenylsilyloxymethyl-2-isopropyloxycarbonyl-7,8,10-trimethoxy-9-methyl-1,2,3,6,11,11a-hexahydropyrazino[1,2-*b*]isoquinolin-4-one (6)

A solution of TBDPSCl (5.6 mL, 21.5 mmol) in dry DCM (350 mL) was added to a stirred mixture of compound **2** (1.6 g, 4.3 mmol) and DMAP (5.25 g, 43 mmol) under argon atmosphere. The mixture was stirred at room temperature, under argon atmosphere for 3.5 h. The reaction mixture was quenched by addition of acetic acid (5.2 mL). After extraction with DCM (30 mL × 3), the combined extracts were washed with H_2O and with a saturated aqueous solution of NaCl, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give a residue, that was purified by flash column chromatography (1:1 hexane/EtOAc) to afford 2 g (71% yield) of **6** as a white solid: mp 63–65 °C; IR (film) ν, 1694, 1634 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ 7.69 (d, J = 6.5 Hz, 2H), 7.55 (d, J = 6.5 Hz, 2H), 7.43 (m, 6H), 6.03 (br s, 1H), 4.96 (m, 1H), 4.45 (m, 1H), 4.11 (dd, J = 10.7 and 3.6 Hz, 1H), 4.02 (m, 1H), 3.98 (m, 1H), 3.95 (m, 2H), 3.73 (s, 3H), 3.70 (s, 3H), 3.65 (s, 3H), 3.52 (m, 1H), 2.90 (dd, J = 16.8 and 4.4 Hz, 1H), 2.67 (dd, J = 16.8 and 11.6 Hz, 1H), 2.19 (s, 3H), 1.27 (s, 6H), 1.04 (s, 9H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 164.7 (C), 154.7 (C), 152.1 (C), 149.9 (C), 146.1 (C), 135.6 (CH), 135.4 (CH), 133.4 (C), 133.2 (C), 129.7 (CH), 129.6 (CH), 127.7 (CH), 127.6 (CH), 124.3 (C), 124.3 (C), 122.9 (C), 69.6 (CH), 65.1 (CH_2), 60.1 (CH_3), 59.9 (CH_3), 59.8 (CH_3), 50.3 (CH), 48.0 (CH_2), 45.1 (CH), 44.4 (CH_2), 27.2 (CH_2), 26.7 (CH_3), 22.1 (CH_3), 19.1 (C), and 9.3 (CH_3). Anal. Calcd for $C_{37}H_{48}N_2O_7Si$: C, 67.24; H, 7.32; N, 4.24. Found: C, 67.12; H, 7.11; N, 4.01.

5.2.5. (6*R**,11a*S**)-6-Hydroxymethyl-7,8,10-trimethoxy-9-methyl-1,2,3,6,11,11a-hexahydro-pyrazino[1,2-*b*]isoquinolin-4-one (7)

To a stirred solution of compound **6** (210 mg, 3.22 mmol) in 0.1 M trifluoroacetic acid (30 mL) was added concd sulfuric acid (1.4 mL) and the resulting solution was stirred at room temperature for 16 h. The reaction mixture was poured into ice, treated with a solution of saturated aqueous solution of NaOH (4 mL), and extracted with DCM (25 mL × 3). The extracts were washed with H_2O and with a saturated aqueous solution of NaCl, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography (EtOAc) to give **7** (1 g, 95%) as an orange solid: mp 82–83 °C; IR (film) ν, 3307, 1681, 1633 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ 5.87 (dd, J = 9.2 and 4.1 Hz, 1H), 3.95 (dd, J = 11.6 and 4.1 Hz, 1H), 3.84 (m, 1H), 3.80 (s, 3H), 3.70 (s, 3H), 3.68 (m, 1H), 3.59 (s, 3H), 3.39 (m, 1H), 3.24–3.21 (m, 2H), 2.93 (dd, J = 10.8 and 2.1 Hz, 1H), 2.83 (dd, J = 16.8 and 4.5 Hz, 1H), 2.67 (dd, J = 16.8 and 11.5 Hz, 1H), 2.10 (s, 3H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 169.5 (C), 152.5 (C), 150.4 (C), 146.6 (C), 125.0 (C), 124.1 (C), 122.8 (C), 63.9 (CH_2), 60.8 (CH_3), 60.4 (CH_3), 60.3 (CH_3), 51.3 (CH), 50.7 (CH_2), 48.4 (CH_2), 47.7 (CH), 28.7 (CH_2), 9.8 (CH_3). Anal. Calcd for $C_{17}H_{24}N_2O_5$: C, 60.70; H, 7.19; N, 8.33. Found: C, 60.62; H, 7.06; N, 8.15.

5.2.6. (6*R**,11a*S**)-6-Hydroxymethyl-2-isopropyloxycarbonyl-7,8,10-trimethoxy-9-methyl-1,2,3,4,11,11a-hexahydro-6*H*-pyrazino[1,2-*b*]isoquinolin-4-carbonitrile (8)

To a stirred solution of crude compound **5** (245 mg, 0.60 mmol) in dry DCM (5 mL) under argon atmosphere was added trimethylsilylcyanide (0.22 mL, 1.65 mmol) and trifluoroboroetherate (0.054 mL, 0.43 mmol). After stirring at −30 °C for 1.5 h, the reaction mixture was quenched by addition of 10 mL of 10% aqueous solution of $NaHCO_3$ and extracted with ethyl acetate (30 mL × 3). The extracts were washed with H_2O and with a saturated aqueous solution of NaCl, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography (EtOAc) to give **8** (205 mg, 78%) as a white solid: mp 74–75 °C; IR (film) ν, 3479, 1698 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ 4.90 (m, 1H), 4.28 (m, 1H), 4.00 (m, 2H), 3.72 (s, 3H), 3.70 (m, 1H), 3.67 (s, 3H), 3.53 (s, 3H), 3.39 (m, 1H), 3.25 (m, 2H), 2.95 (m, 1H), 2.72 (m, 1H), 2.55 (m, 1H), 2.40 (m, 1H), 2.07 (s, 3H), 1.17 (m, 6H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 155.2 (C), 152.2 (C), 149.7 (C), 146.3 (C), 124.7 (C), 123.4 (C), 122.5 (C), 118.8 (C), 69.8 (CH), 61.7 (CH_2), 60.4 (CH_3), 59.9 (CH_3), 59.8 (CH), 59.4 (CH_3), 49.4 (CH), 46.9 (CH_2), 45.8 (CH_2), 45.4 (CH), 22.1 (CH_3), 21.8 (CH_2), 9.2 (CH_3). Anal. Calcd for $C_{22}H_{31}N_3O_6$: C, 60.95; H, 7.21; N, 9.69. Found: C, 60.72; H, 7.16; N, 9.45.

5.3. General procedure to obtain compounds 9 and 10

A 0.1 M solution of **8** (0.4 mmol) in dry DCM, EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 0.81 mmol), DMAP (0.44 mmol) and the corresponding acid compound (0.44 mmol) was stirred under argon atmosphere at room temperature for 21 h. Then, the solvent was evaporated and the residue was solved in EtOAc (50 mL). The organic solution was washed with 0.1 N HCl solution (50 mL), with 10 N aqueous solution of $NaHCO_3$ (50 mL), water and brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo.

5.3.1. (6*R**,11a*S**)-2-Isopropyloxycarbonyl-7,8,10-trimethoxy-9-methyl-6-(1-naphthoyloxymethyl)-1,2,3,4,11,11a-hexahydro-6*H*-pyrazino[1,2-*b*]isoquinolin-4-carbonitrile (9)

The residue was purified by flash chromatography (3:7 hexane/EtOAc) to give **9** (90%) as a white solid: mp 58–60 °C; IR (film) ν, 1704, 1594, 1510 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ 8.65 (d, J = 8.2 Hz, 1H), 7.96 (dd, J = 7.2 and 1.2 Hz, 1H), 7.84 (d, J = 8.2 Hz, 1H), 7.70 (dd, J = 7.6 and 2.2 Hz, 1H), 7.45–7.25 (m, 3H), 4.80 (m, 1H), 4.70 (dd, J = 8.9 and 7.4 Hz, 1H), 4.25 (m, 1H), 4.24 (m, 1H), 4.00 (m, 1H), 3.82 (m, 1H), 3.74 (s, 3H), 3.61 (s, 3H), 3.57 (m, 1H), 3.45 (s, 3H), 3.35 (m, 1H), 3.15 (m, 1H), 3.02 (dd, J = 13.3 and 3.4 Hz, 1H), 2.82 (dd, J = 17.8 and 12.4 Hz, 1H), 2.53 (dd, J = 17.8 and 5.0 Hz, 1H), 2.01 (s, 3H), 1.09 (s, 6H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 167.2 (C), 155.0 (C), 152.3 (C), 149.6 (C), 146.2 (C), 133.7 (C), 133.3 (CH), 131.1 (C), 129.9 (CH), 128.6 (CH), 127.6 (CH), 126.8 (C), 126.2 (CH), 125.5 (C), 124.9 (CH), 124.5 (CH), 123.8 (C), 122.1 (C), 118.8 (C), 68.5 (CH), 63.5 (CH_2), 60.4 (CH_3), 60.0 (CH_3), 59.2 (CH), 58.4 (CH_3), 49.4 (CH), 46.3 (CH_2), 46.2 (CH_2), 45.9 (CH), 22.0 (CH_3), 21.5 (CH_2), 9.8 (CH_3). Anal. Calcd for $C_{33}H_{37}N_3O_7$: C, 67.45; H, 6.35; N, 7.15. Found: C, 67.32; H, 6.26; N, 7.05.

5.3.2. (6*R**,11a*S**)-6-*trans*-Cinnamoylmethyl-2-isopropyloxycarbonyl-7,8,10-trimethoxy-9-methyl-1,2,3,4,11,11a-hexahydro-6*H*-pyrazino[1,2-*b*]isoquinolin-4-carbonitrile (10)

The residue was purified by flash column chromatography (1:1 hexane/EtOAc) to give **10** (90%) as a yellow oil; IR (film) ν, 2252, 1694, 1635 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ 7.75 (d, J = 16.0 Hz, 1H), 7.60 (m, 2H), 7.43 (m, 3H), 6.51 (d, J = 16.0 Hz, 1H), 4.99 (sept, J = 6.4 Hz, 1H), 4.69 (dd, J = 7.6 and 5.8 Hz, 1H), 4.38 (m, 1H), 4.23 (dd, J = 7.6 and 3.3 Hz, 1H), 4.15 (m, 1H), 3.94 (s, 3H), 3.87 (m, 1H), 3.83 (s, 3H), 3.81 (m, 1H), 3.70 (m, 1H), 3.68 (s, 3H), 3.46 (m, 1H), 3.44 (dd, J = 13.2 and 2.9 Hz, 1H), 2.98 (dd, J = 17.8 and 11.4 Hz, 1H), 2.74 (dd, J = 17.8 and 5.2 Hz, 1H), 2.22 (s, 3H), 1.28 (d, J = 6.4 Hz, 6H); ^{13}C NMR (63 MHz, $CDCl_3$) δ

166.5 (C), 155.0 (C), 152.2 (C), 149.6 (C), 146.1(C), 145.1 (CH), 134.1 (CH), 130.4 (CH), 128.8 (CH), 128.1 (CH), 124.9 (C), 123.9 (C), 122.4 (C), 118.7 (C), 117.7 (CH), 69.5 (CH), 63.1 (CH_2), 60.3 (CH_3), 59.9 (CH_3), 59.4 (CH_3), 58.9 (CH), 49.6 (CH), 47.5 (CH_2), 46.4 (CH_2), 46.3 (CH), 22.1 (CH_3), 21.9 (CH_2), 9.3 (CH_3). Anal. Calcd for $C_{31}H_{37}N_3O_7$: C, 66.06; H, 6.62; N, 7.46. Found: C, 66.02; H, 6.54; N, 7.25.

5.4. Biological materials and methods

5.4.1. Cytotoxicity determinations on MDA-MB 231, A-549 and HT-29 cell lines

Cells were placed in 96-well microtiter plates at a density of 5×10^3/well and incubated for 24 h. After that, they were treated with vehicle alone (control) or compounds at the concentrations indicated. One plate from each different cell line was fixed and stained, and used for Tz reference. Treated cells were further incubated for 48 h. To quantify the cytotoxic potential of compounds the sulforhodamine B (SRB) protein stain method was used as follows: cells were washed twice with phosphate-buffered saline (PBS), fixed for 15 min in 1% glutaraldehyde solution, rinsed twice in PBS, and stained in 0.4% (SRB) solution for 30 min at room temperature. Cells were then rinsed several times in 1% acetic acid solution and air-dried. SRB was then extracted in 10 mM trizma base solution and the absorbance measured at 490 nm. Cell survival is expressed as percentage of control cell growth. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma Chemical Co., St. Louis, MO) dye reduction assay in 96-well microplates was used. The assay is dependent on the reduction of MTT by mitochondrial dehydrogenases of viable cell to a blue formazan product, which come be measured spectrophotometrically. Tumor cells were incubated in each well with serial dilutions (5, 2.5, 1, 0.5, 0.1, 0.05, 0.01, and 0.005 μg/mL) of the tested compounds. After two days of incubation (37 °C, 5% CO_2 in a humid atmosphere) 50 μL of MTT (5 mg/mL in PBS) was added to each well and the plate was incubated for a further 2 h (37 °C). The resulting formazan was dissolved in 100 μL DMSO and read at 490 nm. All determinations were carried out in triplicate.

5.4.2. Western blotting and antibodies

For treatment, HT-29 cells were cultured in medium containing 0.5% FBS o.n. After, cells were treated with the specific dose of each compound.

HT-29 cells were lysed as previously described,[30] and 20 μg of protein lysate were resolved in 12% or 15% SDS–PAGE. Antibodies used were: anti p-p53 Ser15, p53 (Cell Signaling, Charlottesville, USA), Phospho-Histone H2A.X Ser139 (Cell Signaling, Charlottesville, USA), Phosho-Chk1/2 Antibody Sampler Kit (Cell Signaling, Charlottesville, USA), Phospho-cdc2 Tyr15, cdc2 (Cell Signaling, Charlottesville, USA), Phospho-Wee1 Ser642, Wee1 (Cell Signaling, Charlottesville, USA), Cyclin B1 (kindly donated by Dr. J. Gonzalez Castaño).

5.4.3. Flow cytometry

HT-29 cells were treated with 5 μg/mL of CDDP and 41 μg/mL of compound **1b** during the indicated times. HT-29 cells were harvested and fixed in 70% ethanol in phosphate-buffered saline (PBS) overnight. For DNA content analysis, the cells were pelleted and re-suspended in PBS containing 100 μg/mL RNase (Qiagen Ltd, Crawley, UK), incubated at room temperature for 30 min. and 125 μg/mL propidium iodide (Sigma–Aldrich, St Louis, MO, USA) and then analyzed using a Beckton Dickinson Flow Cytometer (Cowley, UK). Data were plotted by using CellQuest software; 10,000 events were analyzed for each sample. All experiments were repeated at least three times.

To synchronize in G2/M HT29 cells were treated with 0.2 μg/mL nocodazole overnight, after washed with PBS, and stimulated with 41 μg/mL of compound **1b** or 5 μg/mL of CDDP for 72 h. Population of apoptotic cells was determined by quantifying the population with a DNA content lower than 1 N (SubGo).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.10.007.

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