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Novel 1*H*-pyrrolo[2,3-*b*]pyridine derivatives nortopsentin analogues: synthesis and antitumor activity in peritoneal mesothelioma experimental models

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KEYWORDS: bis-indole alkaloids, CDK1, diffuse malignant peritoneal mesothelioma, paclitaxel, survivin

ABSTRACT

In this study, we describe the synthesis of new nortopsentin analogues, 1*H*-pyrrolo[2,3-*b*]pyridine derivatives, and their biological effects in experimental models of diffuse malignant peritoneal mesothelioma (DMPM), a rare and rapidly fatal disease, poorly responsive to conventional therapies. The three most active compounds, **1f (**3-[2-(5-fluoro-1-methyl-1*H*-indol-3-yl)-1,3-thiazol-4-yl]-1*H*-pyrrolo[2,3-*b*]pyridine), **3f** (3-[2-(1*H*-indol-3-yl)-1,3-thiazol-4-yl]-1-methyl-1*H*-pyrrolo[2,3-*b*]pyridine) and **1l (**3-[2-(5-fluoro-1-methyl-1*H*-indol-3-yl)-1,3-thiazol-4-yl]-1-methyl-1*H*-pyrrolo[2,3-*b*] pyridine), which were shown to act as cyclin-dependent kinase 1 inhibitors, consistently reduced DMPM cell proliferation and induced a caspase-dependent apoptotic response, with a concomitant reduction of the expression of the active Thr^{34}-phosphorylated form of the anti-apoptotic protein survivin. Moreover, the combined treatment of DMPM cells with **3f** derivative and paclitaxel produced a synergistic cytotoxic effect, which was parallel by an enhanced apoptotic response. In the mouse model, i.p. administration of **1f**, **3f**, and **1l** derivatives was effective, resulting in a significant tumor volume inhibition of DMPM xenografts (range, 58%-75%) at well-tolerated doses, and two complete responses were observed in each treatment group.

INTRODUCTION

Diffuse malignant peritoneal mesothelioma (DMPM) is a rare tumor that develops from mesothelial cells that line the peritoneal cavity and accounts for approximately 10-15% of all malignant mesotheliomas.[1,2] Therapeutic approaches proposed thus far for the treatment of DMPM patients (i.e. palliative surgery, radiation therapy and systemic chemotherapy) have not produced satisfactory results and the median patients survival commonly does not exceed 12 months.[3] In the last decade, the introduction of an integrated therapeutic approach combining aggressive cytoreductive surgery (CRS) with hyperthermic i.p. chemotherapy (HIPEC) has significantly improved the median survival,[4] although approximately 40–60% of patients experienced recurrence.[5] For patients not amenable to curative treatment, prognosis remains very poor due to lack of valid alternative therapeutic options.[6] In addition, little is known about the mechanism responsible for the reduced responsiveness to the therapy of DMPM. These considerations emphasize the need to develop novel therapeutic strategies to improve the outcome of DMPM patients.

In recent years, a considerable effort has been made to identify and develop new molecules based on natural compound scaffolds as possible novel cancer therapeutic agents.[7-16] In particular, bis-indole alkaloids represent one of the most important class of pharmaceutically interesting compounds due to their potent biological activities such as antiinflammatory, antimicrobial, antiviral and antitumor.[17-20] Such a class of compounds is characterized by two indole units connected, through their 3 position, by a spacer whose structure can vary from carbocycles or heteocycles differently sized to linear chains. Asterriquinone, whose symmetrical structure involves a quinone moiety, was isolated from *Aspergillus fungi* and showed *in vivo* activity against Ehrlich carcinoma, ascites hepatoma AH13 and mouse P388 leukemia (Chart 1).[21]

Dragmacidins, isolated from a large number of deep water sponges including *Dragmacidon, Halicortex, Spongosorites, Hexadella* and the *tunicate Didemnum candidum*, present different spacers and different related activities. Dragmacidin and dragmacidins A-C, bearing the saturated six membered heterocyclic link piperazine, showed modest cytotoxic activity.[22] Conversely, dragmacidin D, which has a pyrazinone moiety as central core, exhibited several biological properties such as inhibition of serine-threonine protein phosphatases, antiviral, antimicrobial and anticancer activities.[23] Coscinamides, isolated from deep marine sponge *Coscinoderma sp.* bearing a linear chain as a spacer, showed HIV inhibitory activity.[24] Nortopsentins A-C represent the unique group of bis-indolyl alkaloids bearing a five membered ring as spacer, imidazole. They were isolated from *Spongosorites ruetzleri*, and showed *in vitro* cytotoxicity against P388 cells (IC_{50}, 4.5-20.7 μM). Substitution of the indole nitrogen with a methyl group led to derivatives which showed a significant improvement in cytotoxicity against P388 cells relative to that of the parent compounds (IC_{50}, 0.8-2.1 μM).[25-27] The interesting biological activities, of the bis-indolyl marine alkaloids attracted the attention of many researchers who spent much effort in the discovery of new biologically active compounds analogues of these important lead compounds. In this context, dragmacidin analogues bearing as central heterocyclic moiety azine and diazine systems such as pyridine, pyrimidine, pyrazine and pyrazinone were synthesized. Such analogues showed strong inhibitory activity against a wide range of human tumor cell lines.[28-31] Many reports describe the synthesis and the evaluation of the antiproliferative activity of analogues of nortopsentins in which the imidazole ring of the natural compound was replaced by many other five membered heterocycles such as bis-indolyl-thiophenes,[32] pyrazoles,[33] furans,[34] isoxazoles,[34] pyrroles[35] and 1,2,4-thiadiazoles.[36] Many of these analogues showed remarkable antiproliferative activity in cancer cells, often reaching IC_{50}

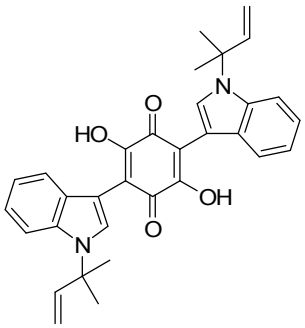
values at sub-micromolar level. In addition, the structural manipulation of the natural nortopsentins -that involved, beside the heterocyclic spacer, one or both indole units- led to the synthesis of 3-[(2-indolyl)-5-phenyl]pyridines and phenylthiazolyl-7-azaindoles. Both these series of compounds showed antiproliferative activity against a wide range of human tumor cell lines at micromolar to sub-micromolar concentrations and were able to inhibit the activity of the cyclin-dependent kinase 1 (CDK1) with IC_{50} values less than 1 μM.[37,38]

Among nortopsentin analogues bearing five-membered heterocycles as spacer were also reported bis indolyl-thiazoles.[28.39] Both papers dealt with the same series of eleven compounds describing the synthesis and, as biological studies, only the antiproliferative tests with IC_{50} values in the micromolar range.

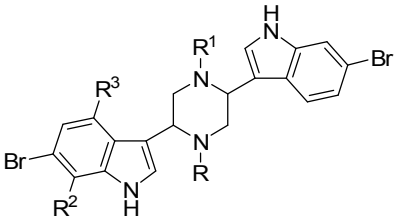
In this paper we report the synthesis of a large number of substituted 3[2-(1*H*-indol-3-yl)-1,3-thiazol-4-yl]-1*H*-pyrrolo[2,3-*b*]pyridines of type **1-3**, nortopsentin analogues in which the spacer is constituted by the thiazole ring and one of the indole units is replaced by a 7-azaindole moiety (CHART 1). Actually, our compounds are similar to the thiazole analogues with the exception of the 7-aza-substitution in one of the indole moieties. However, considering the already mentioned good affinity for CDK1 shown by phenylthiazolyl-7-azaindoles,[38] we thought that the title compounds might increase the interaction with kinases, their putative target.

The cytotoxic activity of all analogues was initially screened in a large panel of human cancer cell lines. In addition, we extensively investigated the effects induced by the three most active compounds, **1f**, **3f** and **1l**, which were shown to act as CDK1 inhibitors, in DMPM experimental models and demonstrated their ability to significantly impair tumor cell proliferation and growth into athymic nude mice.

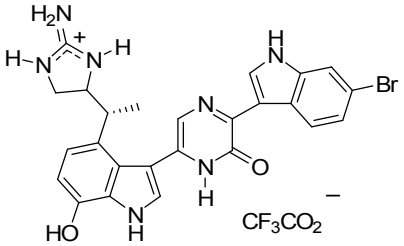
CHART 1. Bis-Indolyl Alkaloids and analogues



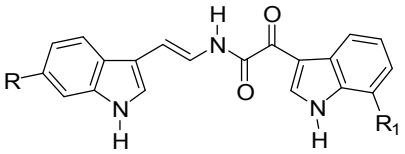
Asterriquinone



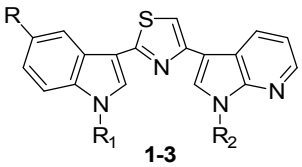
Dragmacidin R=H; R^1=Me; R^2=Br; R^3=OH
Dragmacidin A R=R^2=R^3=H; R^1=Me
Dragmacidin B R=R^1=Me; R^2=R^3=H
Dragmacidin C R=R^1=R^2=R^3=H



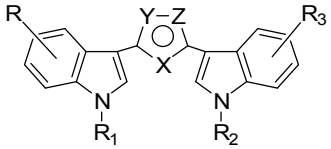
Dragmacidin D



Coscinamide A R=Br; R_1=H
Coscinamide B R=R_1=H
Coscinamide C R=Br; R_1=OH



1-3



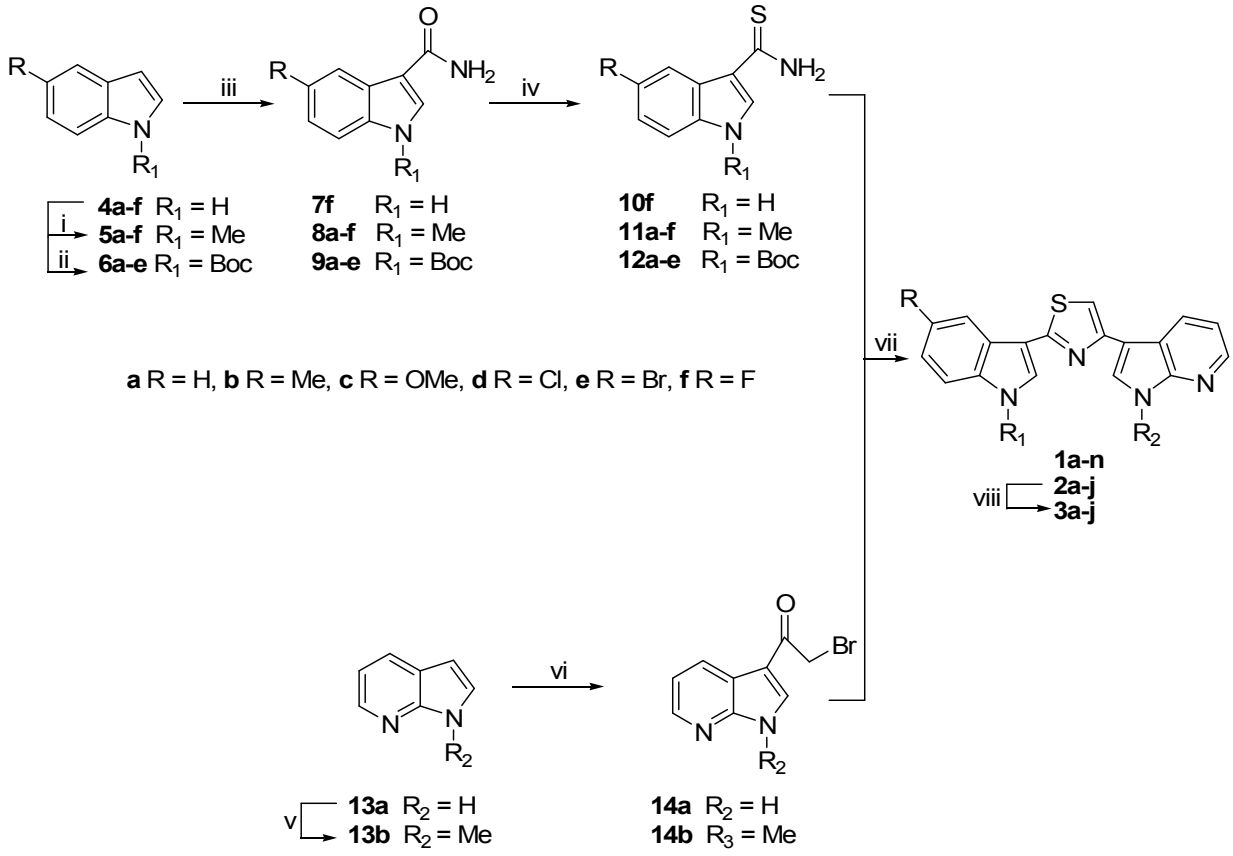
Nortopsentin A X=N; Y=CH; Z=NH; R^1=R^2=H; R=R^3=Br
Nortopsentin B X=N; Y=CH; Z=NH; R=R^1=R^2=H; R^3=Br
Nortopsentin C X=N; Y=CH; Z=NH; R^1=R^2=R^3=H; R=Br
Thiazoles X=N; Y=CH; Z=S; R^1 or R^2=H,Me; R or R^3=H,OMe,Br
Thiophenes X=S; Y=Z=CH; R=R^3=H,Me,OMe,Cl,Br; R^1=R^2=H,Me,SO_2Ph
Pyrazoles X=CH; Y=N; Z=NH,NMe; R=R^3=H,Me,OMe,Cl,Br; R^1=R^2=Me
Furans X=O; Y=Z=CH; R=R^3=H,Me,OMe; R^1=R^2=Me
[1,2]Oxazoles X=CH; Y=N; Z=O; R=R^3=H,Me,OMe,Cl,Br; R^1=R^2=Me
Pyrroles X=NH; Y=Z=CH; R_1=R_2=Me; R=R_3= H,Me,OMe,Cl,Br
1,2,4-Thiadiazoles X=Z=N; Y=S; R or R^3=H,OMe,NO_2,Br; R^1or R^2=H

RESULTS AND DISCUSSION

CHEMISTRY

3-[2-(1*H*-Indol-3-yl)-1,3-thiazol-4-yl]-1*H*-pyrrolo[2,3-*b*]pyridines **1a-n**, **2a-j** and **3a-j** (Table 1) were conveniently prepared by Hantzsch reaction (Scheme 1) between thioamides **10f**, **11a-f** and **12a-e** and α-bromoacetyl compounds **14a,b**.

Scheme 1. Synthesis of substituted 3[2-(1*H*-indol-3-yl)-1,3-thiazol-4-yl]-1*H*-pyrrolo[2,3-*b*]pyridines 1a-n, 2a-j and 3a-j.



Reagents and conditions: for compounds **4b-f**: (i) (a) *t*-BuOK, toluene, TDA-1, rt, 1-24 h; (b) MeI, rt, 0.5-1 h, 96-98%; (ii) $(Boc)_2O$, TEA, THF, reflux, 24-48 h, 90-100%; (iii) (a) CSI, MeCN, 0°C-rt, then rt 0.5-2 h or reflux 15 min; (b) 10% KOH, aq. acetone, 40-70%; (iv)

Lawesson's reagent, toluene or benzene, reflux, 0.5-24 h, 90-98%; (v) (a) *t*-BuOK, benzene, TDA-1, rt, 3 h; (b) CH_3I, rt, 1 h, 96%; (vi) $AlCl_3$, CH_2Cl_2, $BrCOCH_2Br$, reflux, 40 min, 80% for **14a** and 92% for **14b**; (vii) EtOH, reflux, 30 min, 53-95%; (viii) (a) TFA, CH_2Cl_2, reflux, 24 h, (b) aqueous $NaHCO_3$, 54-99%.

The key intermediates of type **10f**, **11a-f** and **12a-e** were synthesized from the corresponding carboxamides **7f**, **8a-f** and **9a-e** easily prepared (40-70%) by reaction of the corresponding indoles **4f**, **5a** (commercially availables), **5b-f** and **6a-e** with chlorosulfonyl isocyanate (CSI) in acetonitrile followed by alkaline hydrolysis of the chlorosulfonyl group. The treatment of amides **7-9** with Lawesson's reagent under reflux in toluene or benzene gave the desired thioamides **10-12** in excellent yields (90-98%). α-Bromoacetyl derivatives **14a,b** were obtained from the corresponding indoles **13a,b** as previously reported.[39] The reaction of thioamides of type **10f**, **11a-f** and **12a-e** with α-bromoacetyl compounds **14a,b** in ethanol under reflux gave the desired 3-[2-(1*H*-indol-3-yl)-1,3-thiazol-4-yl]-1*H*-pyrrolo[2,3-*b*]pyridines **1a-n** and **2a-j** in good to excellent yields (53-95%). The subsequent deprotection of N-t*ert*-butylcarboxylate derivatives **2a-j** using trifluoroacetic acid in dichloromethane under reflux afforded, after neutralization, the corresponding compounds **3a-j** in good to excellent yields (54-99%) (Table 1).

BIOLOGY

1*H*-Pyrrolo[2,3-*b*]pyridine Derivatives Significantly Impair Tumor Cell Growth both *in Vitro* and *in Vivo*. All the synthesized nortopsentin analogues **1a-n**, **2a-j** and **3a-j** were submitted to the National Cancer Institute (Bethesda MD) and pre-screened, at one dose concentration (10^{-5} M), in a panel of 60 cell lines of different tumor types (data not shown).

Compounds **1a, 1d, 1f, 1g, 1l-n, 2f, 3b-f** were further selected for full evaluation at five concentration levels (10^{-4} – 10^{-8} M). The growth inhibition activity of compounds was defined in terms of pGI_{50} value (which represents the -log of the molar concentration of the compound that inhibits 50% net cell growth, GI_{50}). Data reported in the Supporting Table 1 revealed that all the nortopsentin analogues assayed were cytotoxic, and that 9 out of 13 compounds showed GI_{50} values against the total number of cell lines investigated in the micromolar to sub-micromolar range. In spite of the large number of nortopsentin analogues synthesized, the evaluation of the pGI_{50} values did not allow good SAR. However, it was possible to obtain some interesting indications. The most active compound was **3f**, bearing a methyl substituent at the pyrrole nitrogen of the pyrrolo[2,3-*b*]pyridine moiety (azaindole portion). The introduction of a methyl group at the indole moiety maintained the good activity although lower (compare **3f** and **1g**) whereas the further introduction of a fluorine in position 5 of the indole moiety had no significant influence on the biological activity of **1g** (compare **3f**, **1g** and **1l**); conversely the change of the fluorine with chlorine in the same position brought about a decrease of the activity (compare **1f** and **1d**). Replacement of one of the two methyl groups of **1l** with a hydrogen provoked only a very slight decrease of the activity (compare **1l**, **1f** and **1n**). On the contrary, removal of both the methyl groups led to the decrease of the activity of almost one magnitude order (compare **1f** and **1m**). The other components of the bis-demethyl series, **3b-3e**, resulted the less active compounds.

The five most active compounds **1f**, **3f**, **1g**, **1l** and **1n**, which did not show particular selectivity against any of the tumor sub-panels, were further investigated in two additional cell lines, STO and MesoII, derived from human DMPM, a tumor type not included in the NCI panel. Seventy-two-hours exposure to increasing concentrations of each compound resulted in a dose-dependent

inhibition of cell proliferation in both cellular models (Figure 1). As indicated by the drug concentrations required to inhibit growth by 50% (IC_{50} values which ranged from 0.33 to 0.61 μM), compounds **1f**, **3f**, **1g**, **1l** and **1n** exhibited a comparable activity in STO cells (Table 2). By contrast, a variable growth inhibitory effect was induced by the different compounds in MesoII cells (IC_{50} values ranging from 4.11 to 25.12 μM) (Table 2). In addition, while compounds **1f**, **3f**, and **1l** did not interfere with the proliferation of normal cells, compounds **1g** and **1n** displayed an important inhibitory effect on the growth of normal cells (Table 2).

The antitumor activity of **1f**, **3f**, and **1l** derivatives was then evaluated on STO cells xenotransplanted in athymic nude mice. As shown in Figure 2, the treatment with the different compounds resulted in marked tumor growth inhibition. Specifically, at the end of the experiment, a statistically significant tumor volume inhibition (TVI) compared to control (73%, 75% and 58%, for **1f**, **3f**, and **1l** derivatives, respectively) was observed (Table 3). In addition, two complete responses were observed in each treatment group (Table 3). Moreover, the compounds were well tolerated without any appreciable sign of toxicity (Table 3).

1*H*-Pyrrolo[2,3-*b*]Pyridine Derivatives Activity is Mediated by the Inhibition of CDK1 Activity. Since different nortopsentin analogues (i.e. 3-[(2-indolyl)-5-phenyl]pyridines and phenyl-thiazolyl-7-azaindoles) exerted the cellular effects mainly through the inhibition of kinase activity,[38,39] we verified whether compounds **1f**, **3f**, and **1l** could inhibit the *in vitro* catalytic activity of several protein kinases (i.e. CDK1, CDK5, EGFR, FGFR1, RET, MET, KIT, JAK2, PKCA, PKCB, CHK1, MAPK12, SGK, PKA, GSK3α and GSK3β). Nortopsentin derivatives **1f**, **3f**, and **1l** markedly inhibited CDK1 activity, with IC_{50} values (0.89±0.07, 0.75±0.03 and 0.86±0.04 μM, respectively) (Table 4) comparable to those reported for two well-known CDK1

inhibitors, roscovitine and purvalanol A.[39] In addition, our compounds were able to inhibit GSK3β but only at very high concentrations (> 30 μM) (Table 4), whereas they failed to appreciably modify the activity of other protein kinases (IC_{50} values > 50 μM) (data not shown). To confirm the ability of the three selected derivatives to inhibit CDK1 activity in living cells, we assessed the phosphorylation status of histone H1, a specific substrate for CDK1, in STO cells after exposure to **1f**, **3f**, and **1l**. A significant and time-dependent reduction of kinase activity was observed for all the compounds (Figure 3).

Derivatives 1f, 3f, and 1l Impair Cell-Cycle Phase Distribution and Induce Apoptosis by Inhibiting Survivin Activation. Since CDK1/Cyclin B complex is required for G_2 to M transition,[40] we assessed the consequence of nortopsentin derivative treatment on cell cycle progression. Flow cytometry profiles of nuclear DNA content revealed that the treatment of STO and MesoII cells with **1f**, **3f**, and **1l** (at specific IC_{50} of each cell line) was able to induce an accumulation of cells in the G_2/M phase (33.3-45.6% and 41.9-54.3% of the overall cell population, after 48 and 72 h of treatment, respectively), which was parallel by a marked reduction in the percentage of cells in G_1 phase (Figure 4A). Moreover, the percentage of cells accumulated in the sub-G_1 phase significantly increased, with the maximum peak after 72 h of treatment (27.6±1.1%, 25.8±1.2% and 26.5±1.1% of the overall cell population after treatment with **1f**, **3f**, and **1l** in the STO cell line, and 28.6±1.1%, 19.9±1.2% and 27.9±1.1% in the MesoII cell line, respectively) (Figure 4A), suggesting a drug-induced apoptotic response. Consistently, drug-treated STO and MesoII cells showed a significant increase in the number of cells with an apoptotic morphology (in terms of chromatin condensation and DNA fragmentation) (Figure 4B). Specifically, the presence of spontaneous apoptosis was detected

only in a small fraction of control cells (0.5±0.1% and 1.2±0.4% in STO and MesoII cell lines, respectively) but the percentage of apoptotic cells significantly (p<0.01) increased in a time-dependent fashion after treatment with the three derivatives (Figure 4B). In addition, a marked and significant (p<0.02) increase in caspase-9 and caspase-3 catalytic activity (determined *in vitro* by the hydrolysis of specific fluorogenic substrates) was observed after treatment with derivatives **1f**, **3f**, and **1l**. In particular, in STO cells exposed for 72h to derivative **1l** (IC_{80}) the catalytic activity of caspase-9 and caspase-3 was 12- and 10-fold higher, respectively, than that observed in control samples (Figure 4C). Similarly, an 11.9- and 7.8-fold increase in caspase-9 and caspase-3 catalytic activity, respectively, was also observed in MesoII cells (Figure 4C).

We previously reported that one of the main events associated with DMPM chemo-resistance is the dysregulation of apoptotic pathways, mainly due to the over-expression of members of the inhibitors of apoptosis protein (IAP) family, such as survivin,[41] which has essential roles in cell division and apoptosis, and is selectively overexpressed in most human tumors.[42] Based on these findings, we investigated whether one possible mechanism underlying the apoptotic response of DMPM cells after 1*H*-pyrrolo[2,3-*b*]pyridine derivatives exposure was the interference whit survivin activation. In fact, it has been showed that to exert its functions, survivin requires to be phosphorylated on Thr^{34} residue by CDK1/Cyclin B1 complex.[42,43] Previous studies carried out by us and other groups also demonstrated that loss of phosphorylation on Thr^{34}, accomplished through the use of survivin dominant negative mutants or CDK1 inhibitors, destabilizes survivin favoring accelerated clearance and promotes the induction of apoptosis.[42-45] Consistently, results from immunoblotting experiments showed that nortopsentin derivatives-induced apoptosis in DMPM cells occurred as a consequence of a significant and time-dependent reduction of the levels of the active, Thr^{34}-phosphorylated form of survivin (Figure 5).

Derivative 3f Increases Paclitaxel Cytotoxicity in DMPM Cells. Since we previously found that paclitaxel is able to induce survivin phosphorylation on Thr[34],[46] we investigated whether the most active derivative (**3f**) could sensitize DMPM cells to this anticancer agent. To this purpose, STO and MesoII cells were exposed to the taxane for 24 h, followed by a 72 h exposure to derivative **3f**. At all concentrations, **3f** was very effective in cooperating with paclitaxel to inhibit DMPM cell survival (Figure 6A). In fact, when cells were treated with the drugs in combination, an inhibition of the cell proliferation greater than that expected by simple additivity of the effects of the two agents was consistently observed (Figure 6A). Specifically, the synergistic interaction progressively increased by increasing drug concentrations, as indicated by combination index (CI) values, which progressively lowered from 0.81 to 0.42 and from 0.94 to 0.57 in STO and MesoII cells, respectively (Figure 6A). Moreover, the caspase-3 catalytic activity was consistently and significantly ($p<0.001$) higher in cells treated with the combination than in those exposed to each single agent (Figure 6B).

CONCLUSIONS

In this study, we reported the synthesis, the main action mechanism and the preclinical antitumor activity of novel analogues of the marine alkaloid nortopsentin, 1*H*-pyrrolo[2,3-*b*]pyridine derivatives. Newly synthesized nortopsentin derivatives were active against a broad spectrum of human cancer cells lines. However, among the compounds that exhibited the greatest antiproliferative activity in the whole NCI cell line panel, only compounds **1f**, **3f** and **1l** did not appreciably interfere with the proliferation of human normal fibroblasts, suggesting their preferential activity against cancer cells. It is worthy of note that these compounds significantly impaired the growth of two experimental models of DMPM, a rapidly lethal human malignancy, generally refractory to currently available therapeutic options.[2] Since the cellular and molecular bases responsible for the treatment-resistance of DMPM are still poorly understood, the identification of new therapeutic targets and strategies to improve the outcome for DMPM patients are urgently needed.

As far as the mechanisms underlying the antiproliferative effect of **1f**, **3f** and **1l** derivatives were concerned, we showed that the compounds potently inhibited CDK1 activity, and consequently induced a marked time-dependent cell cycle arrest at G_2/M compartment, which was paralleled by an increase in the apoptotic rate, with a concomitant reduction of the phosphorylated form of the anti-apoptotic protein survivin. Moreover, addition of compound **3f** to paclitaxel-treated cells resulted in a synergistic cytotoxic effect, as a consequence of an increased apoptotic response. Most importantly, our results documented a significant anti-tumor activity of the novel nortopsentin derivatives at well-tolerated doses in a DMPM experimental model.

In conclusion, the preclinical evidence that selected nortopsentin derivatives can be active as single agents in a DMPM *in vivo* model, together with the cellular study results showing that

they impair cell cycle progression by inhibiting CDK1 activity and restore the apoptotic response by reducing the active form of the anti-apoptotic protein survivin, provides a solid rationale for the clinical development of these agents in this malignancy.

EXPERIMENTAL SECTION

CHEMISTRY

General Methods

All melting point were taken on a Büchi-Tottoly capillary apparatus and are uncorrected. IR spectra were determined in bromoform with a Jasco FT/IR 5300 spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured at 200 and 50.0 MHz, respectively, in DMSO-d_6 or $CDCl_3$ solution, using a Bruker Avance II series 200 MHz spectrometer. Compounds **1f**, **1l**, **1m**, **1n** were characterized only by ^{1}H NMR spectra, for their poor solubility the ^{13}C spectra were not performed. Column chromatography was performed with Merk silica gel 230-400 mesh ASTM or with Büchi Sepacor chromatography module (prepacked cartridge system). Elemental analyses (C, H, N) were within ± 0.4% of theoretical values and were performed with a VARIO EL III elemental analyzer. Purity of all the tested compounds was greater than 95%, determined by HPLC (Agilent 1100 Series).

General procedure for the synthesis of 5-substituted-1-methyl-1*H*-indoles (5b-f). To a cold solution of indoles **4b-f** (5 mmol) in anhydrous toluene (50 mL), potassium *t*-butoxide (0.76 g, 6.8 mmol) and tris[2-(2-methoxyethoxy)ethyl]amine (TDA-1) (1 or 2 drops) were added. The reaction mixture was stirred at room temperature for 1-24 h and then methyl iodide (0.31 mL, 5 mmol) was added. TLC analysis (dichloromethane/petroleum ether 9/1) revealed that methylation was complete after 0.5-1 h. The solvent was evaporated under reduced pressure. The residue, treated with water, was filtered off and air dried or extracted with dichloromethane (3 x 15 mL), dried (Na_2SO_4) and evaporated to afford the pure methyl derivatives **5b-e**[35] and **5f** (see supporting information).

General procedure for the synthesis of 5-substituted-*tert*-butyl 1*H*-indole-1-carboxylates (6a-e). Di-*tert*-butyl dicarbonate (8.73 g, 40 mmol) and triethylamine (3.3 mL, 23.7 mmol) were added to a solution of appropriate indoles **4a-e** (20 mmol) in tetrahydrofuran (10 mL). The reaction mixture was heated under reflux for 24-48 h. After cooling the solvent was evaporated under reduced pressure and the residue (derivatives **6a** and **6c**) was purified by column chromatography using dichloromethane or dichloromethane /petroleum ether (9/1) as eluent or recrystallized from ethanol (derivatives **6b, 6d, 6e**). For compounds **6a, c-e** see supporting information. Derivatives **6b, 6c** and **6e** were also reported with a different method.[48]

***tert*-Butyl 5-methyl-1*H*-indole-1-carboxylate (6b).** Conditions: 48 h at reflux. Oil; yield: 100%; IR (cm^{-1}) 1732; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.62 (s, 9H, 3xCH_3), 2.39 (s, 3H, CH_3), 6.62 (d, 1H, J = 3.7 Hz, H-3), 7.13 (dd, 1H, J = 8.5, 1.5 Hz, H-6), 7.38 (d, 1H, J = 1.5 Hz, H-4), 7.63 (d, 1H, J = 3.7 Hz, H-2), 7.99 (d, 1H, J = 8.5 Hz, H-7); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 20.8 (q), 27.5 (3xq), 83.3 (s), 107.1 (d), 114.3 (d), 120.8 (d), 125.4 (d), 125.9 (d), 130.4 (s), 131.5 (s), 132.8 (s), 149.0 (s). Anal. Calcd for $C_{14}H_{17}NO_2$: C, 72.70; H, 7.41; N, 6.06. Found: C, 72.40; H, 7.08; N, 6.30.

General procedure for the synthesis of 5-substituted-1-methyl-1*H*-indole-3-carboxamides, 5-substituted-*tert*-butyl 3-carbamoyl-1*H*-indole-1-carboxylates and **5-fluoro-1*H*-indole-3-carboxamide (7f**, **8a-f** and **9a-e)**. To a solution of the appropriate indoles **4f** (0.80 g, 5.9 mmol), **5b-f** or **6a-e** (16 mmol) in anhydrous acetonitrile (20 mL) were added dropwise at 0°C chlorosulfonyl isocyanate (CSI) (0.8 mL, 9.1 mmol for compound **4f** or 1.4 mL, 16 mmol for compounds **5b-f** and **6a-e**). The reaction mixture was warmed to room temperature and stirred for 0.5-2 h (for compounds **5b-f** and **6a-e**) or at reflux for 15 min (for compound **4f)**. A solution of acetone (16 mL) and water (2 mL) was added and the solution was basified using 10%

aqueous solution of potassium hydroxide. The mixture was extracted with ethyl acetate (3 x 20 mL), dried (Na_2SO_4) and the solvent evaporated under reduced pressure. The residue was purified by column chromatography using ethyl acetate (for compounds **7f** and **8a-f**) or dichloromethane /ethyl acetate (1/1) (for compounds **9a-e**) as eluent. For compounds **7f, 8a-e** and **9a,c-e** see supporting information. For compound **8f** analytical and spectroscopic data are previously reported.[49]

***tert*-Butyl 3-carbamoyl-5-methyl-1*H*-indole-1-carboxylate (9b).** Conditions: 2 h after CSI addition. White solid; yield: 60%; mp: 171°C; IR (cm^{-1}) 3336, 3294, 1720, 1664; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.65 (s, 9H, 3xCH_3), 2.41 (s, 3H, CH_3), 7.18 (d, 1H, J = 8.7 Hz, H-6), 7.20 (s, 1H, NH), 7.86 (s, 1H, NH), 7.95 (d, 1H, J = 8.7 Hz, H-7), 8.01 (s, 1H, H-4), 8.42 (s, 1H, H-2); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 21.0 (3xq), 27.6 (q), 84.5 (s), 114.2 (d), 114.7 (s), 121.7 (d), 126.0 (d), 128.4 (d), 128.5 (s), 132.3 (s), 133.0 (s), 148.8 (s), 165.1 (s). Anal. Calcd for $C_{15}H_{18}N_2O_3$: C, 65.68; H, 6.61; N, 10.21. Found: C, 65.61; H, 6.97; N, 9.93.

General procedure for the synthesis of 5-substituted-1-methyl-1*H*-indole-3-carbothioamides, **5-substituted-*tert*-butyl 3-carbamothioyl -1*H*-indole-1-carboxylates** and **5-fluoro-1*H*-indole-3-carbothioamide (10f**, **11a-f** and **12a-e)**. Lawesson's reagent (0.38 g, 0.96 mmol for compound **7f** or 0.80 g, 2 mmol for compounds **8a-f** and **9a-e**) was added to a solution of appropriate derivatives **7f** (0.17 g, 0.95 mmol) or **8a-f** and **9a-e** (4 mmol) in anhydrous toluene or benzene (20 mL). The mixture was heated at reflux under nitrogen atmosphere for 0.5-24 h. After cooling the solvent was evaporated under reduced pressure and the residue was purified by column chromatography using ethyl acetate (for compound **10f**), dichloromethane /ethyl acetate (98/2) (for compounds **11a-f**) or dichloromethane (for compounds **12a-e**) as eluent. For compounds **10f, 11a-f** and **12a,c-e** see supporting information.

***tert*-Butyl 3-carbamothioyl-5-methyl-1*H*-indole-1-carboxylate (12b).** Conditions: benzene as solvent, 1 h at reflux. Orange solid; yield: 98%; mp: 130°C; IR (cm^{-1}) 3479, 3365, 1737, 1597; ^{1}H NMR (200 MHz, DMSO-d_6) δ:1.64 (s, 9H, 3xCH_3), 2.42 (s, 3H, CH_3), 7.20 (d, 1H, J = 8.7 Hz, H-6), 7.97 (d, 1H, J = 8.7 Hz, H-7), 8.27 (s, 1H, H-2), 8.39 (s, 1H, H-4), 9.31 (s, 1H, SH), 9.52 (s,1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 21.2 (q), 27.6 (3xq), 84.7 (s), 114.3 (d), 120.2 (s), 122.2 (d), 126.1 (d), 126.3 (d), 127.9 (s), 132.3 (s), 133.4 (s), 148.7 (s), 192.9 (s). Anal. Calcd for $C_{15}H_{18}N_2O_2S$: C, 62.04; H, 6.25; N, 9.65. Found: C, 61.80; H, 6.63; N, 9.73.

General procedure for the synthesis of derivatives (1a-n and 2a-j). A suspension of the appropriate derivatives **10-12** (5 mmol) and **14** (5 mmol) in anhydrous ethanol (20 mL) was heated under reflux for 30 min. The precipitate, obtained after cooling, was filtered off, dried and crystallized with ethanol to afford derivatives **1a-n** and **2a-j**. For compounds **1a-n** and **2a,c-j** see supporting information.

***tert*-Butyl 5-methyl-3-[4-(1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)-1,3-thiazol-2-yl]-1*H*-indole-1-carboxylate (2b).** Yellow solid; yield: 60%, mp; 199-201°C; IR (cm^{-1}) 3303, 1788; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.68 (s, 9H, 3xCH_3), 2.51 (s, 3H, CH_3), 7.31 (dd, 1H, J = 8.8, 1.4 Hz, H-6'), 7.41 (dd, 1H, J = 7.9, 5.0 Hz, H-5''), 7.99 (s, 1H, H-2'), 8.06 (d, 1H, J = 8.8 Hz, H-7'), 8.24-8.30 (m, 3H, H-2'', H-4' and H-5), 8.43 (dd, 1H, J = 5.0, 1.3 Hz, H-6''), 8.85 (d, 1H, J = 7.9 Hz, H-4''), 12.40 (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 21.1 (q), 27.6 (3xq), 84.7 (s), 108.4 (s), 109.7 (s), 110.6 (d), 112.1 (d), 114.5 (d), 116.5 (d), 120.9 (d), 124.3 (s), 125.5 (d), 127.0 (d), 127.3 (d), 130.0 (s), 133.2 (s), 135.0 (s), 139.1 (d), 144.1 (s), 148.8 (s), 149.4 (s), 160.2 (s). Anal. Calcd for $C_{24}H_{22}N_4O_2S$: C, 66.96; H, 5.15; N, 13.01. Found: C, 66.67; H, 5.41; N, 13.28.

General procedure for the synthesis of derivatives (3a-j). To a suspension of appropriate derivative **2a-j** (0.78 mmol) in dichloromethane (10 mL) trifluoacetic acid (1.1 mL) was added. The reaction was heated at reflux for 24 h. The mixture was neutralized with saturated aqueous sodium hydrogen carbonate solution. The solvent was dried (Na_2SO_4), evaporated under reduced pressure and the residue recrystallized with ethanol to afford derivatives **3a-j**. For compounds **3a,c-j** see supporting information.

3-[2-(5-Methyl-1*H*-indol-3-yl)-1,3-thiazol-4-yl]-1*H*-pyrrolo[2,3-*b*]pyridine (3b). Green solid; yield: 95%; mp: 243-244°C; IR (cm^{-1}) 3303, 3337; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.51 (s, 3H, CH_3), 7.10 (d, 1H, *J* = 8.4 Hz, H-6'), 7.44 (d, 1H, *J* = 8.4 Hz, H-7'), 7.58 (dd, 1H, *J* = 7.6, 4.6 Hz, H-5''), 7.92 (s, 1H, H-2'), 8.07 (s, 1H, H-2''), 8.23-8.36 (m, 2H, H-4' and H-5) 8.55 (d, 1H, *J* = 4.6 Hz, H-6''), 9.16 (d, 1H, *J* = 7.6 Hz, H-4''), 11.8 (s, 1H, NH), 12.90 (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 21.6 (q), 108.7 (d), 109.6 (s), 111.1 (s), 112.1 (d), 116.2 (d), 119.7 (d), 121.0 (s), 124.1 (d), 124.5 (s), 126.8 (d), 127.2 (d), 129.7 (s), 134.9 (d), 135.0 (s), 137.4 (d), 142.1 (s), 147.5 (s), 163.0 (s). Anal. Calcd for $C_{19}H_{14}N_4S$: C, 69.07; H, 4.27; N, 16.96. Found: C, 68.79; H, 4.56; N, 16.69.

BIOLOGY

Drugs. Paclitaxel was purchased from Santa Cruz Biotechnology. The nortopsentin derivatives were prepared as described above. To obtain a 50 μM stock solution, each compound was completely dissolved in 1% dimethylsulfoxide (DMSO), stored at -20°C protected from light, and then diluted in complete culture medium immediately before use at the appropriate concentration.

Cell Culture. Human DMPM cell lines (STO and MesoII) were established from surgical specimens of patients admitted to Fondazione IRCCS Istituto Nazionale dei Tumori of Milan as previously described.[43] Their origin was authenticated through microsatellite analysis by the AmpFISTR Identifiler PCR amplification kit (Applied Biosystem, PN4322288). The normal human lung fibroblast cell line (WI38) was obtained from the American Type Culture Collection (ATCC). Cells were tested for the absence of Mycoplasma fortnightly and maintained in the logarithmic growth phase as a monolayer in appropriate culture media supplemented with 10% heat-inactivated fetal bovine serum in a humidified incubator at 37°C with a supply of 5% CO_2/95% air atmosphere.

Cell Proliferation Assay. After harvesting in the logarithmic growth phase, 4500 cells /50 µl were plated in 96-well flat-bottomed microtiter plates (EuroClone) for 24 h and then treated with increasing concentrations of derivatives **1f**, **3f**, and **1l** (0.1–100 µM) for 72 h. Control cells received vehicle alone (DMSO). In the combination experiments, STO and MesoII cells were treated with paclitaxel for 24h and then exposed to **3f** for additional 72h. All studies were performed in eight replicates and repeated at least three times independently. At the end of drug exposure, the antiproliferative potential was determined with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, purchased from Promega) according to the manufacturer's protocols. Optical density was read at 490 nm on a microplate reader and the results were expressed as a percentage, relative to DMSO-treated cells. Dose-response curves were created and IC_{50} and IC_{80} values (i.e., concentrations able to inhibit cell growth by 50% and 80%, respectively) were determined graphically from the curve for each compound.

The method described by Chou&Talalay[47] was used to determine the nature of the interaction between paclitaxel and **3f**. Drugs were combined at a constant ratio of paclitaxel and **3f**

concentrations (1:100 and 1:333 in STO and MESOII, respectively). The type of interaction was assessed in terms of combination index (CI). CI values of <1 or >1 indicated synergy or antagonism, respectively, whereas a CI value of 1 indicates additivity.

***In Vitro* Kinase Assay.** Drug effect on protein kinase activity was evaluated using the OmniaTM® Recombinant Kit (Invitrogen) according to the manufacturer's protocols. Briefly, increasing concentrations of **1f**, **3f**, and **1l** (0.05-50 μM) were mixed to recombinant CDK1/cyclin B, CDK5/p25, EGFR, FGFR1, RET, MET, KIT, JAK2, PKCA, PKCB, CHK1, MAPK12, SGK, PKA, GSK3α and GSK3β proteins (Invitrogen) in 1X kinase buffer containing a peptide substrate, ATP, and dithiothreitol, and the kinase reaction was performed at 30°C for 30min. Fluorescences were measured upon excitation at 360 nm and emission at 485 nm. IC_{50} values (drug concentration at which enzyme activity is reduced by 50%) were determined graphically from the curve obtained for each compound.

CDK1 Activity Assay in Cells. Cellular lysates (0.5 mL) were combined with the monoclonal antibody anti-Cyclin B1 (Abcam Inc.) in the presence of 100 μL of a mixture of A-Sepharose 20% (v/v) protein (GE Healthcare) and lysis buffer (10mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton X-100, 1mM PMSF, 5μg/mL Aprotinin e 20μg/mL Leupeptin). The samples were immunoprecipitated for 4 h at 4˚C and then incubated with 3μg of Histone H1 (Upstate Biotechnology Inc.) for 20 min at 30°C in a specific buffer containing 20mM Tris-HCI, pH 7.5, 10mM $MgCl_2$, 5pM cold ATP, and 10μCi of [γ32P] ATP. The reaction was stopped adding an equal volume of loading buffer with SDS and then analyzed by 12% polyacrylamide gel. Resolved bands were quantified by densitometric analysis using the Image-Quant software (Molecular Dynamics).

Cell Cycle Phase Distribution and Apoptosis Analyses. Both adherent and floating cells were fixed in 70% EtOH and incubated at 4°C for 30 min in staining solution containing 50μg/mL of propidium iodide, 50mg/mL of RNase, and 0.05% Nonidet-P40 in PBS. Samples were analyzed with a FACS-Calibur cytofluorimeter (Becton Dickinson). At least 30000 events were read and histograms were analyzed using the CellQuest software according to the Modfit model (Becton Dickinson).

For apoptosis analysis, an aliquot of propidium-stained cells was scored for nuclear morphology of apoptosis (chromatin condensation and DNA fragmentation) by fluorescence microscopy. The percentage of cells with an apoptotic nuclear morphology was determined by scoring at least 500 cells in each sample. In the same cellular samples, the catalytic activity of caspase-9 and caspase-3 was measured as the ability to cleave the specific substrates N-acetyl-Leu-Glu-His-Asp- pNA (LEHD-pNA) and N-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA) by means of the APOPCYTO/caspase-9 and APOPCYTO/caspase-3 kits (MBL), respectively, according to manufacturer's instructions. The hydrolysis of the specific substrates was monitored by spectrofluorometry with 380-nm excitation and 460-nm emission filters. Results were expressed as relative fluorescence units (rfu).

Immunoprecipitation and Western Blot Analysis. To assess the phosphorylation status of the anti-apoptotic protein survivin, total protein extract (250μg) was immunoprecipitated with the anti-human survivin antibody (Abcam Inc.) for 16 h at 4°C with a mixture of A-Sepharose 20% (v/v) protein (GE Healthcare) and lysis buffer. After separation by 15% SDS gel electrophoresis, samples were transferred to nitrocellulose. The filters were blocked in PBS with 5% skim milk and incubated overnight with the primary antibody specific for Thr^{34}-phosphorylated survivin (Abcam Inc.). The filters were then incubated with the secondary anti-rabbit peroxidase-linked

antibody (GE Healthcare). Bound antibodies were detected using the SuperSignals West PICO chemiluminescent substrate (Pierce Biotechnology Inc.). The results were quantified by densitometric analysis using the Image-Quant software (Molecular Dynamics).

***In Vivo* Experiments.** Female athymic Swiss nude mice (8-10 weeks-old, purchased from Charles River) were maintained in laminar flow rooms keeping temperature and humidity constant. Mice had free access to food and water. Experiments were approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale Tumori of Milan according to institutional guidelines that are in compliance with national and international laws and policies. Exponentially growing STO cells were subcutaneously injected into mouse right flank (10^7cells/flank). Eight mice for each group experimental group were used. All compounds were dissolved in a mixture of DMSO (10%), Cremophor EL (5%) and saline solution (85%) and delivered i.p. every day a week for three weeks (qdx4-5/wx3w) starting from the day after cell inoculum. Tumor growth was followed by biweekly measurements of tumor diameters with a Vernier caliper. Tumor volume (TV) was calculated according to the formula: TV (mm3) = d2xD/2 where d and D are the shortest and the longest diameter, respectively. The efficacy of the drug treatment was assessed as: *i)* Tumor volume inhibition percentage (TVI %) in treated *versus* control mice, calculated as: TVI% = 100-(mean TV treated/mean TV control x 100), and *ii)* Complete regression (CR), i.e. disappearance of the tumor lasting until the end of experiment. The toxicity of the drug treatment was determined as body weight loss and lethal toxicity. Deaths occurring in treated mice before the death of the first control mouse were ascribed to toxic effects.

Statistical Analysis. Statistical evaluation of data was done with two-tailed Student's t test. *P*s < 0.05 were considered statistically significant.

SUPPORTING INFORMATION

Table 1 and spectroscopic data (IR, ^{1}H, ^{13}C NMR) and elemental analysis for compounds **5f, 6a,c-e**, **7f, 8a-e**, **9a,c-e**, **10f**, **11a-f**, **12a,c-e, 1a-n**, **2a,c-j** and **3a,c-j**.

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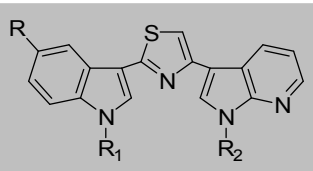
TABLES

Table 1. 3-[2-(1*H*-indol-3-yl)-1,3-thiazol-4-yl]-1*H*-pyrrolo[2,3-*b*]pyridines 1a-n, 2a-j, 3a-j.



Compd	R	R_1	R_2	Yields	Compd	R	R_1	R_2	Yields
1a	H	Me	H	85%	**2d**	Cl	Boc	H	90%
1b	Me	Me	H	70%	**2e**	Br	Boc	H	80%
1c	OMe	Me	H	90%	**2f**	H	Boc	Me	92%
1d	Cl	Me	H	78%	**2g**	Me	Boc	Me	53%
1e	Br	Me	H	60%	**2h**	OMe	Boc	Me	90%
1f	F	Me	H	95%	**2i**	Cl	Boc	Me	90%
1g	H	Me	Me	75%	**2j**	Br	Boc	Me	72%
1h	Me	Me	Me	60%	**3a**	H	H	H	99%
1i	OMe	Me	Me	85%	**3b**	Me	H	H	95%
1j	Cl	Me	Me	57%	**3c**	OMe	H	H	99%
1k	Br	Me	Me	55%	**3d**	Cl	H	H	99%
1l	F	Me	Me	91%	**3e**	Br	H	H	54%
1m	F	H	H	87%	**3f**	H	H	Me	71%
1n	F	H	Me	60%	**3g**	Me	H	Me	99%
2a	H	Boc	H	85%	**3h**	OMe	H	Me	90%
2b	Me	Boc	H	60%	**3i**	Cl	H	Me	99%
2c	OMe	Boc	H	90%	**3j**	Br	H	Me	90%

Table 2. Cytotoxic activity of compounds 1f, 3f, 1g, 1l and 1n in DMPM and normal cells.

	IC_{50} (μM)[a]		
Compd	**STO**	**MesoII**	**WI38**
1f	0.49±0.07	25.12±3.06	>100
3f	0.33±0.07	4.11±0.22	>100
1g	0.61±0.14	16.77±1.99	18.76±3.21
1l	0.43±0.11	4.85±0.64	>100
1n	0.54±0.09	13.27±0.74	15.44±3.87

[a] Data are reported as IC_{50} values (concentration of drug required to inhibit growth by 50%) determined by MTS assay after 72 h of continuous exposure to each compound. The data represents mean values ± SD of at least three independent experiments.

Table 3. Activity of derivatives 1f, 1l and 3f on STO cells xenotransplanted in athymic nude mice.

Compd	TVI (%)[a]	CR[b]	BWL (%)[c]	TOX[d]
1f	73*	2/8	4	0/8
1l	75**	2/8	1	0/8
3f	58*	2/8	7	0/8

[a] Tumor volume inhibition (%) in treated *vs* control mice, determined 17 days after the end of drug treatment (day 35). [b] Complete response, disappearance of tumor induced by treatment. [c] BWL, body weight loss induced by treatment (%). [d] Toxic death on treated animals. $^{**}p<0.01$, $^{*}p<0.05$

Table 4. *In vitro* kinase inhibitory properties of derivatives 1f, 1l and 3f.

	IC_{50} (μM)[(a)]		
Protein kinase	**1f**	**1l**	**3f**
CDK1	0.89±0.07	0.75±0.03	0.86±0.04
GSK-3β	42.18±3.28	40.18±2.94	35.68±1.69

[a] Concentration of drug required to inhibit by 50% (IC_{50}) the activity of CDK1 e GSK-3β. Values represent the mean values ± SD of three independent experiments.

FIGURE LEGENDS

Figure 1. Effects of **1f** (▲), **3f** (○), **1g** (□), **1l** (■) and **1n** (●) derivatives on cell proliferation of STO and MesoII cell lines exposed for 72 h to increasing concentrations of each compound. Data are expressed as percentage values with respect to non-treated cells (only DMSO) and represent the mean values ±SD (standard deviation) of three independent experiments.

Figure 2. Activity of **1f**, **3f**, and **1l** derivatives on STO cells xenotransplanted on athymic mice. Drugs were administered ip at 25 (**1f** and **1l**) or 50 (**3f**) mg/kg qdx4-5/wx3w, starting from the day after the injection. $^{**}p<0.01$, $^{*}p<0.05$

Figure 3. Effect of **1f**, **3f**, and **1l** derivatives on CDK1 kinase activity in DMPM cells. (A) Representative kinase assay illustrating the CDK1 activity in STO cells at different intervals after exposure to 1% (v/v) DMSO (control cells; C) or to derivative **3f** (IC_{50}; T). (B) Densitometric quantification of CDK1 activity in STO cells exposed to derivatives **1f**, **3f**, and **1l** for 24, 48 and 72 h. CDK1 activity was performed by immunoprecipitation and kinase assay as described in Materials and Methods section. Data are reported as the percentage of CDK1 activity in cells exposed to derivatives **1f**, **3f**, and **1l** (gray column) compared with DMSO-treated cells (black column) and represent the mean values ± SD of at least three independent experiments. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$.

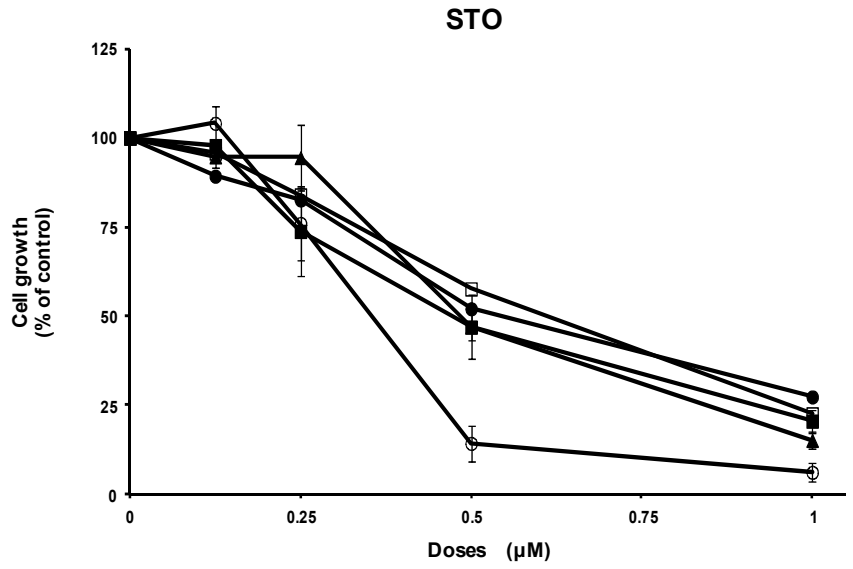
Figure 4. Effect of **1f**, **3f**, and **1l** derivatives on cell-cycle progression and apoptosis induction. (A) STO and MesoII cells were exposed to 1% (v/v) DMSO (control cells; C) or to derivatives **1f**, **3f**, and **1l** (IC_{50}) for 48 and 72h, and the effect on cell cycle distribution was assessed as

described in Materials and Methods section. Data are reported as the percentage of cells in sub-G_1 (dark gray), G_1 (light gray), S (black), and G_2/M (white) phases and represent the mean values of three independent experiments; SDs were always within 5%. (B) The percentage of cells with an apoptotic morphology was assessed by fluorescence microscopy after exposure of DMPM cells to 1% (v/v) DMSO (control cells; C) or to derivatives **1f**, **3f**, and **1l** at 48 h (black column) and 72 h (gray column) after treatment. Data are expressed as mean values ± SD of at least three independent experiments. $^{**}p<0.001$, $^{*}p<0.01$. (C) The catalytic activity of caspases was assessed after a 72-h of exposure of DMPM cells to 1% (v/v) DMSO (control cells; C) or to derivatives **1f**, **3f**, and **1l**. Caspase-9 (black column) and caspase-3 (gray column) catalytic activity was determined *in vitro* by hydrolysis of the fluorogenic substrates (LEHD-pNA and DEVD-pNA, respectively). Data are expressed as mean values ± SD of at least three independent experiments. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.02$.

Figure 5. Effect of **1f**, **3f**, and **1l** derivatives on survivin phosphorylation. (A) Representative western blotting illustrating the survivin phosphorylation status in STO cells after exposure to 1% (v/v) DMSO (control cells; C) or to derivative **3f**, (IC_{50}; T). (B) Densitometric quantification of survivin phosphorylation levels in STO cells exposed to **1f**, **3f**, and **1l** for 24, 48 and 72 h. The phosphorylation of survivin on Thr^{34} residue was evaluated on STO cells treated with 1% (v/v) DMSO (control cells; black column) or to derivatives **1f**, **3f**, and **1l** (IC_{50}; gray column) by Western immunoblotting. Survivin was immunoprecipitated using the anti-human survivin antibody and analyzed with the antibody to phosphorylated Thr^{34} as described in Materials and Methods section. Data are expressed as mean values ± SD of at least three independent experiments. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$.

Figure 6. Cytotoxic effect of **3f** derivative in combination with paclitaxel in DMPM cells. (A) The cytotoxic effect of paclitaxel and **3f** derivative, alone or in combination, was assessed by MTS assay as described in Materials and Methods section. The dashed lines represent the expected additive effect of the combination, calculated as the product of the effects of the individual drugs. Data are expressed as mean values ± SD of at least three independent experiments. CI was calculated according to Chou&Talalay.[47] (B) Caspase-3 catalytic activity was determined *in vitro* by the hydrolysis of the specific fluorogenic substrate (DEVD-pNA). Data are expressed as mean values ± SD of at least three independent experiments. *p<0.001.

FIGURE 1


STO
Cell growth (% of control)
125
100
75
50
25
0
0
0.25
0.5
0.75
1
Doses (μM)

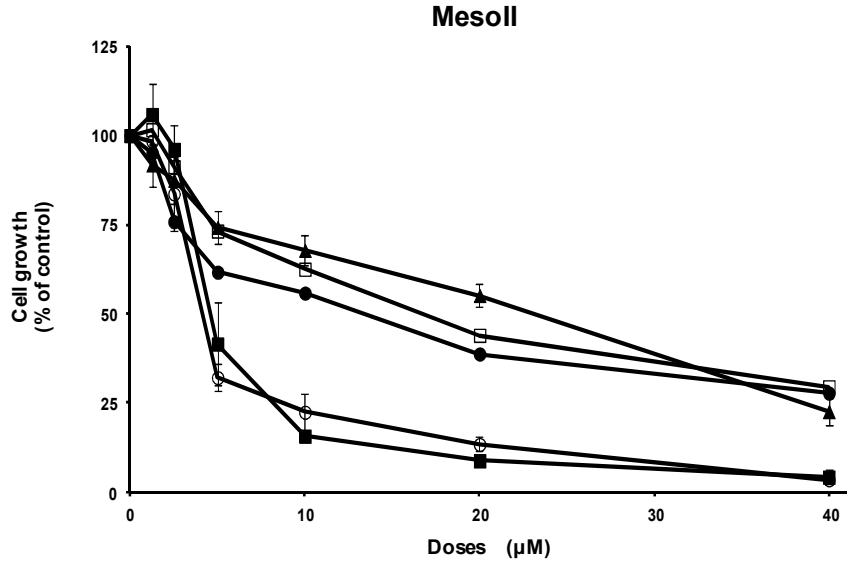

MesoII
Cell growth (% of control)
125
100
75
50
25
0
0
10
20
30
40
Doses (μM)

FIGURE 2

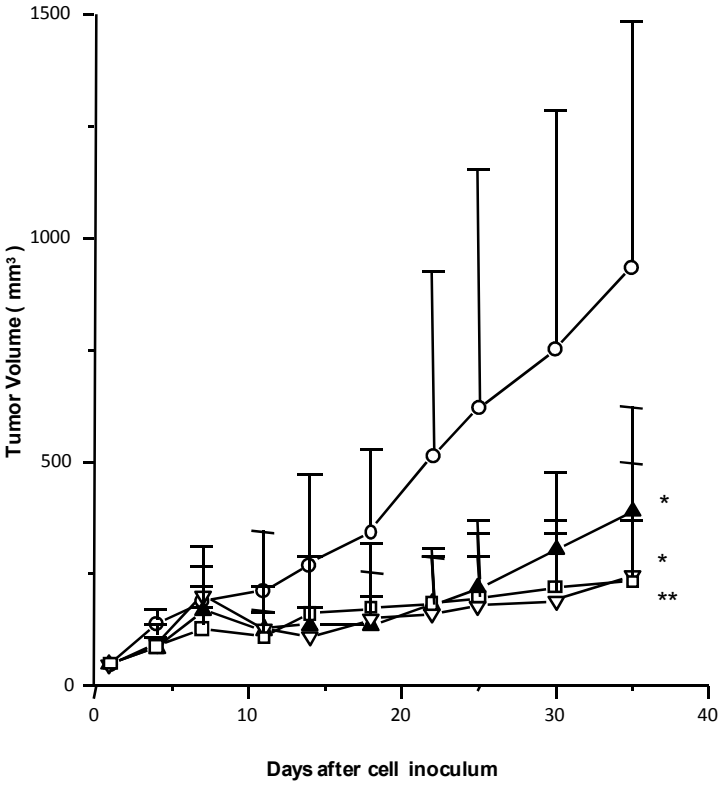

1500
1000
500
0
Tumor Volume (mm³)
0
10
20
30
40
Days after cell inoculum
*
*
**

FIGURE 3

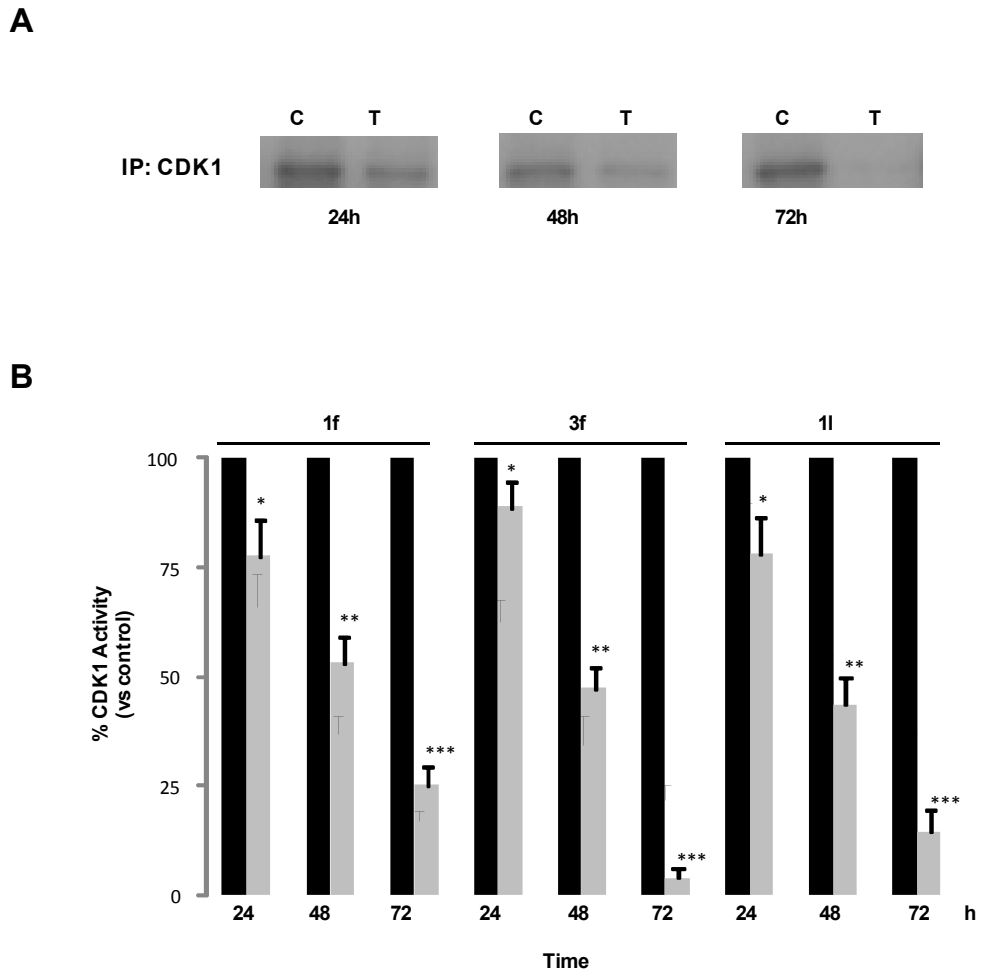

A
C
T
C
T
C
T
IP: CDK1
24h
48h
72h
B
1f
3f
1l
100
75
50
25
0
% CDK1 Activity
(vs control)
*
**
***
24
48
72
h
Time

FIGURE 4

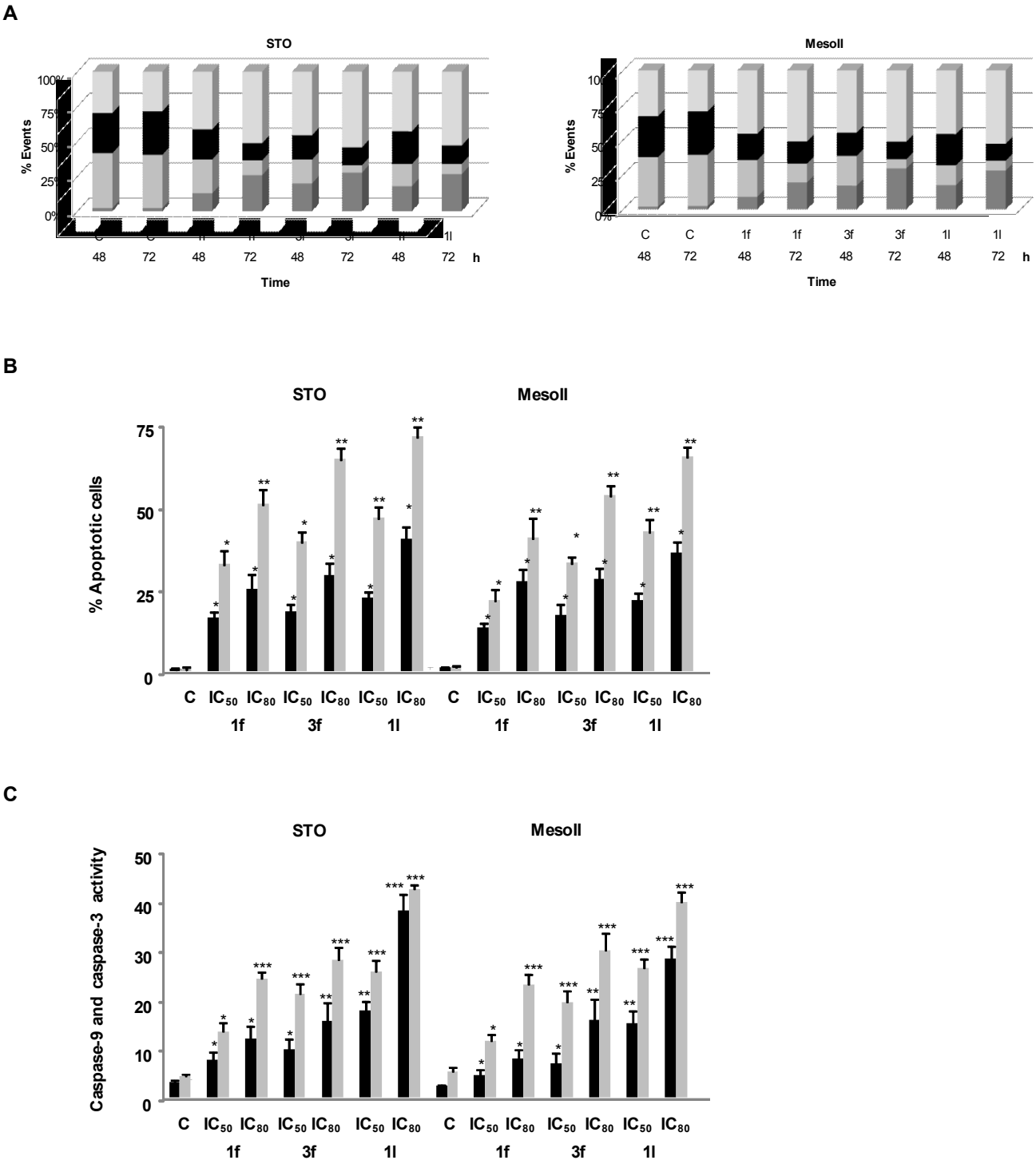

A
STO
Mesoll
% Events
100%
75%
50%
25%
0%
C C 1f 1f 3f 3f 1l 1l
48 72 48 72 48 72 48 72 h
Time
B
STO
Mesoll
% Apoptotic cells
75
50
25
0
C IC50 IC80 IC50 IC80 IC50 IC80 C IC50 IC80 IC50 IC80 IC50 IC80
1f 3f 1l 1f 3f 1l
C
STO
Mesoll
Caspase-9 and caspase-3 activity
50
40
30
20
10
0
C IC50 IC80 IC50 IC80 IC50 IC80 C IC50 IC80 IC50 IC80 IC50 IC80
1f 3f 1l 1f 3f 1l

FIGURE 5

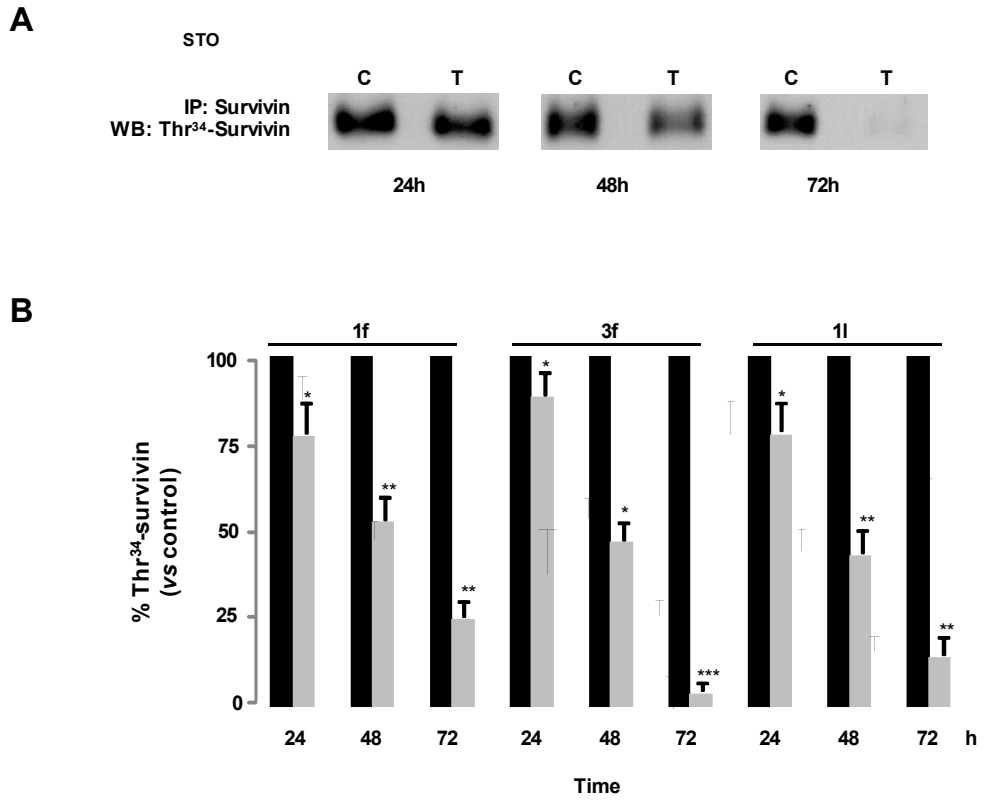
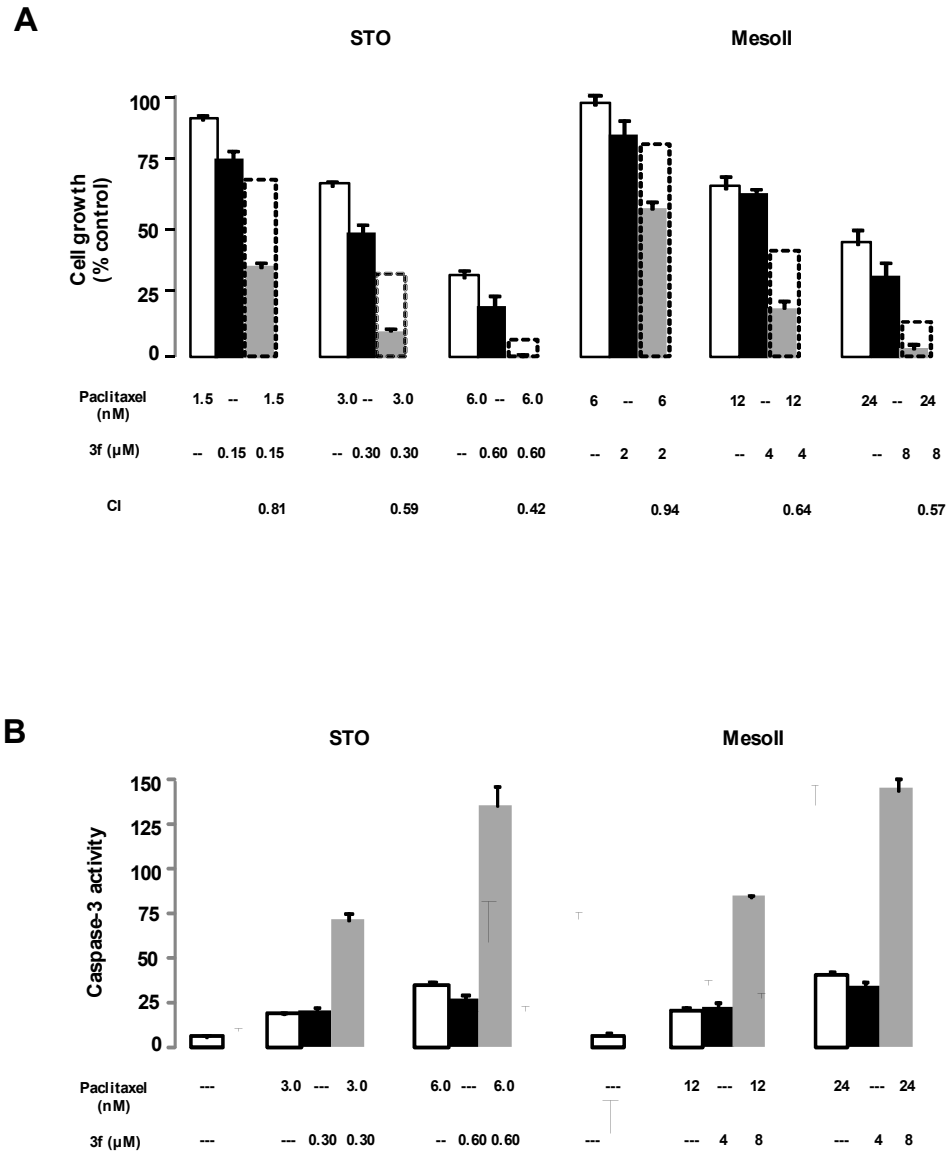

A
STO
C
T
C
T
C
T
IP: Survivin
WB: Thr34-Survivin
24h
48h
72h
B
1f
3f
1l
100
75
50
25
0
% Thr34-survivin
(vs control)
*
**
**
*
*
***
*
**
**
24
48
72
24
48
72
24
48
72
h
Time

FIGURE 6


A
STO
Mesoll
Cell growth (% control)
100
75
50
25
0
Paclitaxel (nM)
1.5 -- 1.5 3.0 -- 3.0 6.0 -- 6.0 6 -- 6 12 -- 12 24 -- 24
3f (μM)
-- 0.15 0.15 -- 0.30 0.30 -- 0.60 0.60 -- 2 2 -- 4 4 -- 8 8
CI
0.81 0.59 0.42 0.94 0.64 0.57
B
STO
Mesoll
Caspase-3 activity
150
125
100
75
50
25
0
Paclitaxel (nM)
--- 3.0 --- 3.0 6.0 --- 6.0 --- 12 --- 12 24 --- 24
3f (μM)
--- --- 0.30 0.30 -- 0.60 0.60 --- --- 4 8 --- 4 8