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Design, Synthesis and Biological Evaluation of 3,5-Disubstituted 2-Amino Thiophene Derivatives As a Novel Class of Antitumor Agents

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Abstract: In search of new compounds with strong antiproliferative activity and simple molecular structure, we designed a novel series of agents based on the 2-amino-3-alkoxycarbonyl/cyano-5-arylethylthiophene scaffold. The presence of the ethyl spacer between the 2',5'-dimethoxyphenyl and the 5-position of the thiophene ring, as well as the number and location of methoxy substitutents on the phenyl ring, played a profound role in affecting the antiproliferative activity. Among the synthesized compounds, we identified the 2-amino-3-cyano-[2-(2,5-dimethoxyphenyl)ethyl] thiophene **2c** as the most promising derivative against a wide panel of cancer cell lines (IC_{50}=17-130 nM). The antiproliferative activity of this compound appears to correlate well with its ability to inhibit tubulin assembly and the binding of colchicine to tubulin. Moreover **2c**, as determined by flow cytometry, strongly induced arrest in the G2/M phase of the cell cycle, and annexin-V and propidium iodide staining indicate that cell death proceeds through an apoptotic mechanism that follows the intrinsic mitochondrial pathway.

Keywords: tubulin, thiophene, anticancer agents, colchicine site, apoptosis.

1. Introduction

Microtubules are one of the three components of the cytoskeleton and are involved in a wide range of cellular functions critical for the life cycle of the cell. These include most notably cell division, where they form the mitotic spindle formation required for proper chromosomal separation.[1-3] The microtubule system is also important in other fundamental cellular processes, such as regulation of motility, cell signaling, formation and maintenance of cell shape, secretion and intracellular transport.[4] Research oriented toward the discovery of naturally occurring and synthetic molecules that bind to tubulin and disrupt microtubule

dynamics have attracted considerable attention in the last few decades, since microtubules are a validated and important pharmacological target in cancer chemotherapy.[5-8]

Among synthetic small molecule tubulin inhibitors, Novartis Pharmaceuticals Corporation identified a new chemical entity as an anticancer agent, named SDZ LAP 977 (**1**, Chart 1). This compound contains two fragments, the methyl ester of salicylic acid and, at its 5-position, a 2',5'-dimethoxyphenylethyl moiety.[9-10] Compound **1** is active at low micromolar concentrations as an antiproliferative agent against both human pancreatic tumor (MIA PaCa-2) and epithelial carcinoma (A431) cells, blocking the cell cycle in mitosis through inhibition of tubulin polymerization.

The classical bioisosteric equivalence between benzene and thiophene prompted us to synthesize a series of 2-amino-3,5-disubstituted thiophene derivatives with general formula **2**, in which a 2-aminothiophene system bearing at its 3-position a methoxycarbonyl, ethoxycarbonyl or cyano group replaced the salicylic acid methyl ester of compound **1**. In this regards, replacement of an hydroxyl with an amino group furnished encouraging results in terms of significantly increased cytotoxicity against many human cancer lines in a series of benzophenone-type CA-4 analogues named phenstatins.[11]

Keeping constant the 2',5'-dimethoxyphenylethyl pharmacophore of **1** at the C-5 position of the thiophene ring, compounds **2a-c** were designed in order to probe the importance of the substituent at the C-3 position, by the introduction of methoxycarbonyl (**2a**), ethoxycarbonyl (**2b**) and cyano (**2c**) groups. By the synthesis of 3,4-disubstituted derivatives **2d** and **2e**, we also evaluated the effect on activity caused by the concomitant presence of a methyl or ethoxycarbonyl at the C-4 position of the thiophene core in compounds **2a** and **2b**, respectively.

Compounds **2f-m** represent a second series of molecules in which the importance for antiproliferative activity caused by the absence (**2m**) or presence of a single methoxy substitution at the 2'- or 4'-position of the phenyl group of the phenylethyl moiety was evaluated (compounds **2fik** and **2ghj**, respectively).

In order to study whether the ethyl spacer between the two aromatic ring systems was also beneficial for activity, we also synthesized compounds **2n-o**, in which the 2',5'-dimethoxyphenyl nucleus was directly attached to the C-5 position of thiophene ring.

We examined the efficacy of the newly synthesized compounds on a panel of cancer cell lines and, in addition, the mechanism of action of the most active compound was investigated in detail.

Chart 1 should be inserted here

2. Chemistry

The general strategy for the preparation of compounds **2a-o** is shown in Scheme 1. Crucial intermediates were identified as the thiophene derivatives **5a-e**, obtained in a two step procedure starting from the 2-amino thiophenes **3a-e**.[12] These latter compounds were converted in good yields to the phthalimido derivatives **4a-e** using phthalic anhydride in refluxing acetic acid. The subsequent regioselective bromination of **4a-e** in a mixture of acetic acid and sodium acetate using bromine furnished intermediate 5-bromothiophenes **5a-e**. Compounds **6a** and **6b** were prepared by palladium-mediated coupling chemistry of 2-methoxycarbonyl and 2-cyano thiophenes **5a** and **5c**, respectively, with 2', 5'-dimethoxybenzeneboronic acid under heterogeneous conditions [$Pd(PPh_3)_4$, K_2CO_3] in refluxing toluene. Removal of the *N*-protected pthaloyl group was performed with hydrazine in refluxing ethanol to afford **2n** and **2o**.

The building block intermediates **5a-e**, coupled by the Sonogashira cross-coupling reaction with the appropriate terminal arylacetylene[13] in the presence a catalytic amount of $PdCl_2(PPh_3)_2$ and CuI in a mixture of TEA and DMF, afforded the arylacetylenic intermediates **7a-m**, which were reacted with hydrazine in refluxing ethanol to furnish compounds **8a-m**. The reduced analogues **2a-m**, characterized by the presence of a flexible ethyl linker, were prepared starting from derivatives **8a-m** by catalytic hydrogenation of the acetylenic triple bond to ethyl over 10% Pd/C.

Scheme 1 should be inserted here

3. Biological Results and Discussion

3.1. *In vitro* antiproliferative activities. Table 1 summarizes the growth inhibitory effects of derivatives **2a-o** and reference compound **1** against murine leukemia (L1210), human cervix carcinoma (HeLa), murine mammary carcinoma (FM3A/0) and human T-lymphoblastoid (Molt4/C8 and CEM/0) cells. Only compounds **2a-c**, with a common 2-amino-5-[2-(2,5-dimethoxy-phenyl)-ethyl]-thiophene structure, were more active than **1** against FM3A cells. With the exception of this cell line, **2a** was less active than **1** in the other cell lines, while **2b** had potency similar to that of **1** in two of the three cell lines studied. In our hands, **1** was about 30-fold less active in the FM3A cells than the other cell lines examined, and, with the exceptions of **2a-c**, none of the other compounds we prepared was as active as **1** in any cell line where comparisons could be made. Aside from the extraordinary activity of **2c**, in comparison with **1**, a submicromolar IC_{50} value was only observed with compound **2g** in CEM cells. We did examine a fifth cell line (Molt4/C8) with the new compounds, but not **1**, and submicromolar IC_{50} values were obtained with **2a**, **2f-h** and **2j**. However, it is only **2c** that had especially noteworthy antiproliferative activity in these cell lines, ranging from 35 nM in

the CEM cells to 130 nM in the HeLa cells. It was therefore far superior to the lead compound **1**. Based on compounds **2a-e**, this activity appears to derive from the nitrile substituent at position C-3 of the thiophene ring. Additional, much less active compounds with this substituent (**2k-m** and **2o**) provide interesting SAR information. Removal of one (**2k**) or both (**2m**) methoxy groups from the phenyl ring lead to substantial losses of antiproliferative activity, greater with the removal of both methoxy groups. A single methoxy group at a different position (*para* to the ethylthiophene moiety, **2l**) also resulted in a much less active compound. Finally, elimination of the two carbon bridge between the ring systems (**2o**) almost eliminated antiproliferative activity.

In summary, using the biospheric thiophene ring to replace the second phenyl ring of compound **1**, we synthesized a potent new agent with amino and nitrile substituents at positions C-2 and C-3, respectively. Thus far, additional manipulations of the basic structure of compound **2c** have only resulted in much less active compounds.

Table 1 should be inserted here

3.2. Effect of compound 2c on a panel of human cancer cell lines. To further characterize the tumor cell growth inhibitory profile of **2c**, it was tested against a panel of an additional twelve human cancer cell lines. These studies confirmed its excellent activity, with IC_{50} values obtained ranging from 12 to 86 nM (Table 2). The greatest activity was observed with Jurkat, the least activity with MT4 both two T-leukemia cell lines.

Table 2 should be inserted here

3.3. Inhibition of tubulin polymerization and colchicine binding. To investigate whether the antiproliferative activity of the highly active **2c** derived from an interaction with tubulin, **2c** and additional compounds were evaluated for their inhibition of tubulin polymerization

(Table 3).[14] For comparison, compound **1** was examined in contemporaneous experiments. In the assembly assay, compounds **2c** was the most active compound (IC_{50} value 0.74 μM), with activity 3-fold greater than that of **1** (IC_{50}, 2.0 μM). Compounds **2a** and **2b** inhibited tubulin assembly with IC_{50} values of 1.9 and 3.1 μM, respectively. The other compounds examined were much less active as inhibitors of tubulin assembly. Note that **2c** had much greater antiproliferative activity than **1**, **2a** and **2b**, despite the similar effects on tubulin assembly.

Such discrepancies in antitubulin versus antiproliferative activity are not infrequently observed, but the reasons are usually uncertain, as is the case here. Among possible explanations is that we are using bovine brain tubulin in the former studies, and its composition in terms of tubulin isotypes differs significantly from that of different human cancer cell lines.[15]

The most active compounds in the *in vitro* tubulin polymerization assay were also evaluated for their effects on the binding of [^{3}H]colchicine to tubulin.[16] In this assay, derivative **2c** potently inhibited the binding of [^{3}H]colchicine to tubulin, and with 84% inhibition was 1.5-fold more active than **1**, which in this experiment inhibited colchicines binding by 52%. Inhibition of colchicine binding by compounds **2a** and **2b** was also lower than inhibition by **2c**, but comparable to inhibition by **1**. For the highly active **2c**, a good correlation was observed between antiproliferative activity, inhibition of tubulin polymerization and inhibition of colchicine binding. These results indicate that an interaction with tubulin is a major cause of the antiproliferative activity of this group of compounds.

Table 3 should be inserted here

3.4. Molecular Modeling. A series of molecular modeling simulations were performed to investigate the structural basis of the findings described above. The *in* silico studies were

focused on understanding the role of the substitution pattern on the phenyl ring as well as the replacement of the ester with a nitrile group in the binding of these analogues to tubulin. Initially, we performed a series of molecular docking calculations, which resulted in a suggested binding mode for this class of compounds very similar to the one reported in literature for several other colchicines site compounds.[17] In particular, all the compounds were placed with the phenyl ring in proximity of βCys241 and with the amino group within hydrogen-bond distance of αThr179. However, as shown in Fig. 1, the docking software also predicted virtually the same binding pose for inactive compounds like **2f**, and therefore our initial modeling studies did not provide a plausible justification for the empirical data obtained. For this reason, we performed a set of molecular dynamics (MD) simulations on the complexes between tubulin and three representative compounds of the series **2a**, **2c** and **2f**. The results obtained gave more detailed insight into a possible binding mechanism for these molecules. The most active compound in the series, **2c**, was stable during the 3ns MD, with the phenyl ring always interacting with the βCys241 (see Supplementary Data) and forming a stable hydrogen bond with αThr179 (82% rate of formation). Furthermore, the 2-aminothiophene moiety maintained a close contact with βAsn258 (Fig. 2). Compound **2a** also maintained a close contact with βCys241, but the hydrogen bond with αThr179 was less stable (22% rate of formation). Interestingly, the interaction of **2a** with βAsn258 seemed to be affected, possibly because of increased steric hindrance by the ester methyl group. Finally, in the case of **2f**, the binding pose was significantly less stable (see Supporting Information). During the MD simulation, the compound moved away from both βCys241 and αThr179 (12% rate of formation of the hydrogen bond with the amino group of **2f**). The interaction with βAsn258 was also disrupted during the calculations. In summary, from the MD simulations it appears that the methoxy group in the *meta* position of the phenyl ring is

important in locking the compounds into the binding pocket, while the stability of the hydrogen bond of the 2-aminothiophene moiety with αThr179, supported by the interaction with βAsn258, is responsible for enhancing the activity of **2c** relative to **2a** and **2f**.

Figures 1 and 2 should be inserted here

3.5. Analysis of cell cycle effects of compound 2c. The effects of different concentrations of compound **2c,** after 24 h of treatment on cell cycle progression were examined in HeLa cells (Figure 3, Panel A) and in Jurkat cells (Figure 3, Panel B). The compound caused a significant G2/M arrest in a concentration-dependent manner, with a rise in G2/M cells occurring at a concentration as low as 30 nM, while at higher concentrations more than 80% of the cells were arrested in G2/M, with a concomitant decrease of cells in the other phases of the cell cycle (G1 and S).

We next studied the association between **2c**-induced G2/M arrest and alterations in expression of proteins that regulate cell division. Cell arrest at the prometaphase/metaphase to anaphase transition is normally regulated by the mitotic checkpoint.[18] The cdc2/cyclin B complex controls both entry into and exit from mitosis. Phosphorylation of cdc2 on Tyr15 and phosphorylation of cdc25C phosphatase on Ser216 negatively regulate the activation of the cdc2/cyclin B complex.

Thus, dephosphorylation of these proteins is needed to activate the cdc2/cyclin B complex. cdc25C is a major phosphatase that dephosphorylates the site on cdc2 and autodephosphorylates itself. Phosphorylation of cdc25C directly stimulates both its phosphatase and autophosphatase activities, a condition necessary to activate cdc2/cyclin B on entry of cells into mitosis.[19-20] As shown in Figure 3 (panel C) in HeLa cells, **2c** at low concentrations (50 and 100 nM) caused a marked increase in cyclin B1 expression after both 24 and 48 h treatments. In addition, slower migrating forms of phosphatase cdc25C appeared

at 24 h, indicating changes in the phosphorylation state of this protein, followed by the disappearance of both forms of cdc25C at 48 h. The phosphorylation of cdc25C directly stimulates its phosphatase activity, and this is necessary to activate cdc2/cyclin B on entry of cells into mitosis.[19,20] In addition, we observed a dramatic de-phosphorylation of cdc2 (Tyr15), just after 24 h of treatment, which should result in inhibition of formation of the cdc2/cyclinB complex.

Figure 3 should be inserted here

3.6. Compound 2c induces apoptosis. To characterize the mode of cell death induced by **2c**, a biparametric cytofluorimetric analysis was performed using PI, which stains DNA and enters only dead cells, and fluorescent immunolabeling of the protein annexin-V, which binds to PS in a highly selective manner.[21] Dual staining for annexin-V and with PI permits discrimination between live cells (annexin-V^-/PI^-), early apoptotic cells (annexin-V^+/PI^-), late apoptotic cells (annexin-V^+/PI^+) and necrotic cells (annexin-V^-/PI^+) (Figure 4, Panel A). As depicted in Figure 4 (Panel B), after 24 h of treatment with compound **2c** at different concentrations, we observed a significant, accumulation of annexin-V positive cells in a concentration–dependent manner that further increased at 48 h (Panel C) even at the lowest concentration used (15 nM).

Figure 4 should be inserted here

3.7. Effect of 2c on mitochondrial depolarization and ROS generation. Mitochondria play an essential role in the propagation of apoptosis.[22] It is well established that, at an early stage, apoptotic stimuli alter the mitochondrial transmembrane potential ($\Delta\psi_{mt}$). $\Delta\psi_{mt}$ was monitored by the fluorescence of the dye JC-1. In untretated cells (high $\Delta\psi_{mt}$), JC-1 displays a red fluorescence (590 nm). This is caused by spontaneous and local formation of aggregates

that are associated with a large shift in emission. In contrast, when the mitochondrial membrane is depolarized (low $\Delta\psi_{mt}$), JC-1 forms monomers that emit at 530 nm. As shown in Figure 5 (Panel A), **2c** in HeLa cells induced a time and concentration–dependent increase in the proportion of cells with depolarized mitochondria.

Mitochondrial membrane depolarization is associated with mitochondrial production of ROS.[23] Therefore, we investigated whether ROS production increased after treatment with **2c**. We utilized two fluorescence indicators, HE and H_2-DCFDA, as previously described.[24] The results presented in Figure 5 (Panels B and C) showed that **2c** induced the production of significant amounts of ROS in comparison with control cells, which agrees with the previously described dissipation of $\Delta\psi_{mt}$. Altogether, these results indicate that this compound induced apoptosis through the mitochondrial pathway.

Figure 5 should be inserted here

3.8. Compound 2c induces caspase activation and reduced expression of anti-apototic proteins. Caspases, which are proteolytic enzymes, are the central executioners of apoptosis, and their activation is mediated by various inducers.[25] Synthesized as proenzymes, caspases are themselves activated by specific proteolytic cleavage reactions. Caspases-2, -8, -9, and -10 are termed apical caspases and are usually the first to be activated in the apoptotic process. Following their activation, they in turn activate effector caspases, in particular caspase-3.[26] Following treatment of HeLa cells with compound **2c** (50 and 100 nM), we observed an early activation of caspase-9 at 24 h, followed at 48 h by activation of caspase-3 and of the caspase-3 substrate PARP (Figure 6, Panel A). These results are in good agreement with the mitochondrial depolarization described above, indicating that **2c** induces the apoptotic intrinsic (mitochondrial) apoptosis pathway.

We also examined whether the induction of apoptosis by **2c** was associated with changes in the expression of two proteins of the Bcl-2 family (Bcl-2 and Mcl-1), since there is increasing evidence that they share the signaling pathways induced by antimicrotubule compounds.[27-29] Our results showed (Figure 6, Panel B) that the expression of the anti-apoptotic proteins Bcl-2 and Mcl-1 was remarkably reduced after a 24 h and disappeared after a 48 h treatment at both concentrations used. In addition, expression of the X-linked inhibitor of apoptosis protein (XIAP), a member of the apoptosis inhibitors protein family (IAP), was strongly reduced concomitant with caspase activation (Figure 6, Panel B), suggesting that **2c**-induced apoptosis may also be mediated by XIAP down-regulation.

Figure 6 should be inserted here

4. Conclusions

SDZ LAP 977 is an inhibitor of tubulin polymerization which consists of two segments, the methyl ester of salicylic acid and 2',5'-dimethoxybenzene, linked by an ethyl spacer. The salicylic acid methyl ester and 2',5'-dimethoxy benzene fragments were varied structurally to furnish a new series of molecules based on the 2-aminothiophene scaffold, in which substitution at the C-3 or C-5 positions play important roles in potency and selectivity against a panel of cancer cell lines. Replacement of salicylic acid methyl ester of compound **1** with a 2-amino-3-cyanothiophene system resulted in the identification of a novel potent antiproliferative agent (**2c**) against a panel of human and murine tumor cell lines, with IC_{50} values ranging from 35 to 130 nM. The antiproliferative activity of **2c** was enhanced by 3-230-fold with respect to **1** in four cell lines. **2c** was also clearly superior to its methoxycarbonyl (**2a**) and ethoxycarbonyl (**2b**) analogues. Removing the ethyl spacer between the two aromatic rings of **2c** to furnish derivative **2o** resulted in a substantial

reduction of activity. We also demonstrated that the antiproliferative activity of **2c** required the presence of the 2',5'-dimethoxyphenylethyl moiety. We identified tubulin as the molecular target of compounds **2a-c**, since they inhibited tubulin assembly and the binding of colchicine to tubulin. In particular, derivative **2c** was the most potent inhibitor of tubulin polymerization and of colchicine binding in this group of compounds. In keeping with tubulin as its target, **2c** induced cell cycle arrest in the G2/M phase of the cell cycle. This mitotic arrest was accompanied by an increase of cyclin B expression and a decrease in intracellular levels of cdc2 (Tyr15). Our data demonstrated that **2c** induced apoptosis through the intrinsic mitochondrial pathway with caspase activation. Moreover, the compound strongly reduced the expression of Bcl-2 and Mcl-1, an anti-apoptotic member of the Bcl-2 family, which recently has been reported to regulate sensitivity to antimitotic drugs.[29] These findings indicate that **2c** is endowed with high antiproliferative activity and should be further investigated to determine its therapeutic potential.

5. Experimental Section

5.1.Chemistry.

5.1.1. Materials and Methods. ^{1}H spectra were recorded on a Bruker AC 200 or a Varian 400 Mercury Plus spectrometer. Chemical shifts (δ) are given in ppm, and the spectra were recorded in appropriate deuterated solvents, as indicated. Positive-ion electrospray ionization (ESI) mass spectra were recorded on a double-focusing Finnigan MAT 95 instrument with BE geometry. Melting points (mp) were determined on a Buchi-Tottoli apparatus and are uncorrected. All products reported showed ^{1}H NMR spectra in agreement with the assigned structures. The purity of tested compounds was determined by combustion elemental analyses conducted by the Microanalytical Laboratory of the Chemistry Department of the University

of Ferrara with a Yanagimoto MT-5 CHN recorder elemental analyzer. All tested compounds yielded data consistent with a purity of at least 95% as compared with the theoretical values. All reactions were carried out under an inert atmosphere of dry nitrogen, unless otherwise indicated. Standard syringe techniques were used for transferring dry solvents. Reaction courses and product mixtures were routinely monitored by TLC on silica gel (precoated F_{254} Merck plates), and compounds were visualized with aqueous $KMnO_4$. Flash chromatography was performed using 230-400 mesh silica gel and the indicated solvent system. Organic solutions were dried over anhydrous Na_2SO_4. 2',5'-dimethoxyphenylboronic acid and arylacetylenes are commercially available and used as received. All chemicals and reagents were purchased from Aldrich (Sigma-Aldrich) or Lancaster (Alfa Aesar, Johnson Matthey Company).

5.2. General procedure A for the synthesis of compounds 4a-e. To a suspension of thiophene derivatives **3a-e** (5 mmol) in acetic acid (30 mL) was added phthalic anhydride (890 mg, 6 mmol). After stirring for 18 h at reflux, the solvent was evaporated and the residue dissolved in CH_2Cl_2 (50 mL). The organic solution was washed with a saturated solution of $NaHCO_3$ (15 mL), water (10 mL) and brine (10 mL). The organic layer was dried and evaporated, and the residue was purified by flash chromatography on silica gel or by crystallization with petroleum ether to afford **4a-e.**

5.2.1. Methyl 2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)thiophene-3-carboxylate (4a). Following general procedure A, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **4a** as a white solid (56% yield); mp 147-149 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.72 (s, 3H), 7.36 (d, *J*=5.6 Hz, 1H), 7.53 (d, *J*=5.6 Hz, 1H), 7.81 (m, 2H), 7.98 (m, 2H). MS (ESI): $[M+1]^+$=288.3.

5.2.2. Ethyl 2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)thiophene-3-carboxylate (4b). Following general procedure A, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **4b** as a pink solid (58% yield); mp 67-69 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.07 (t, *J*=7.0 Hz, 3H), 4.16 (q, *J*=7.0 Hz, 2H), 7.35 (d, *J*=5.6 Hz, 1H), 7.55 (d, *J*=5.6 Hz, 1H), 7.81 (m, 2H), 7.98 (m, 2H). MS (ESI): $[M+1]^+$=302.2.

5.2.3. 2-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)thiophene-3-carbonitrile (4c). Following general procedure A, the crude residue purified by crystallization from ethyl ether, furnished **4c** as a green solid (88% yield); mp 165-167 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.29 (d, *J*=5.6 Hz, 1H), 7.44 (d, *J*=5.6 Hz, 1H), 7.86 (m, 2H), 8.02 (m, 2H). MS (ESI): $[M+1]^+$=255.1.

5.2.4. Methyl 2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-4-methylthiophene-3-carboxylate (4d). Following general procedure A, the crude residue purified by crystallization from petroleum ether, furnished **4d** as a white solid (74% yield); mp 129-130 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.46 (s, 3H), 3.68 (s, 3H), 7.02 (s, 1H), 7.82 (m, 2H), 8.00 (m, 2H). MS (ESI): $[M+1]^+$=302.2.

5.2.5. Diethyl 2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)thiophene-3,4-dicarboxylate (4e). Following general procedure A, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **4e** as a yellow oil (74% yield). ^{1}H-NMR ($CDCl_3$) δ: 1.24 (m, 6H), 4.39 (m, 4H), 7.82 (m, 2H), 7.93 (s, 1H), 7.97 (m, 2H). MS (ESI): $[M+1]^+$=374.3.

5.3. General procedure B for the preparation of compounds 5a-e. To an acetic acid solution (12 mL) of derivatives **4a-e** (5 mmol) containing sodium acetate (451 mg, 5.5 mmol), bromine (800 mg, 0.36 mL, 5 mmol) was added dropwise with stirring at room temperature

over 5 min. After 1 h, acetic acid was removed under *vacuo*, and the crude residue was diluted with EtOAc (25 mL), washed successively with an aqueous 10% sodium thiosulphate solution (10 mL), a saturated solution of $NaHCO_3$ (10 mL), water (5 mL) and brine (5 mL), and dried and concentrated. The resulting residue was purified by crystallization from petroleum ether or by column chromatography on silica gel.

5.3.1. Methyl 5-bromo-2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)thiophene-3-carboxylate (5a). Following general procedure B, the crude residue purified by crystallization from petroleum ether, furnished **5a** as a grey solid (55% yield); mp 135-137 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.73 (s, 3H), 7.51 (s, 1H), 7.83 (m, 2H), 7.96 (m, 2H). MS (ESI): $[M+1]^+$=366.0 and 368.0.

5.3.2. Ethyl 5-bromo-2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)thiophene-3-carboxylate (5b). Following general procedure B, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 1:9 (v:v) for elution, furnished **5b** as a white solid (87% yield); mp 109-110 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.09 (t, *J*=7.2 Hz, 3H), 4.15 (q, *J*=7.2 Hz, 2H), 7.52 (s, 1H), 7.82 (m, 2H), 7.98 (m, 2H). MS (ESI): $[M+1]^+$=380.1 and 382.1.

5.3.3. 5-Bromo-2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)thiophene-3-carbonitrile (5c). Following general procedure B, the crude residue purified by crystallization from petroleum ether, furnished **5c** as a yellow solid (82% yield); mp 204-205 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.88 (m, 2H), 8.00 (s, 1H), 8.03 (m, 2H). MS (ESI): $[M+1]^+$=333.0 and 335.0.

5.3.4. Methyl 5-bromo-2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-4-methylthiophene-3-carboxylate (5d). Following general procedure B, the crude residue purified by crystallization from petroleum ether, furnished **5d** as a grey solid (80% yield); mp 152-154 °C. ^{1}H-NMR

($CDCl_3$) δ: 2.42 (s, 3H), 3.68 (s, 3H), 7.85 (m, 2H), 7.96 (m, 2H). MS (ESI): $[M+1]^+$=380.1 and 382.2.

5.3.5. Diethyl 5-bromo-2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)thiophene-3,4-dicarboxylate (5e). Following general procedure B, the crude residue purified by crystallization from petroleum ether, furnished **5e** as a white solid (75% yield), mp 101-103 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.07 (t, *J*=7.4 Hz, 3H), 1.38 (t, *J*=7.2 Hz, 3H), 4.15 (q, *J*=7.4 Hz, 2H), 4.39 (q, *J*=7.4 Hz, 2H), 7.82 (m, 2H), 7.97 (m, 2H). MS (ESI): $[M+1]^+$=452.1 and 454.1.

5.4. General procedure C for the synthesis of compounds 6a and 6b. A mixture of thiophene derivatives **5a** or **5c** (0.5 mmol), K_2CO_3 (104 mg, 0.75 mmol, 1.5 equiv.), 2,5-dimethoxyphenylboronic acid (182 mg, 1 mmol, 2 equiv.) and $Pd(PPh_3)_4$ (13.5 mg, 0.012 mmol) in dry toluene (10 mL) was stirred at 100 °C under nitrogen for 18 h, cooled to room temperature, filtered through celite and the filtrate evaporated *in vacuo*. The residue was dissolved with dichloromethane (15 mL), and the resultant solution was washed sequentially with 5% $NaHCO_3$ (5 mL), water (5 mL) and brine (5 mL). The organic layer was dried over Na_2SO_4, evaporated and the residue purified by flash column chromatography on silica gel.

5.4.1. Methyl 5-(2,5-dimethoxyphenyl)-2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-thiophene-3-carboxylate (6a). Following general procedure C, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **6a** as a white solid (58% yield); mp 201-202 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.74 (s, 3H), 3.85 (s, 3H), 3.89 (s, 3H), 6.93 (m, 2H), 7.22 (d, *J*=5.2 Hz, 1H), 7.82 (m, 2H), 7.86 (s, 1H), 7.97 (m, 2H). MS (ESI): $[M+1]^+$=424.2.

5.4.2. 5-(2,5-Dimethoxyphenyl)-2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)thiophene-3-carbonitrile (6b). Following general procedure C, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **6b** as a yellow solid (55% yield); mp 205-207 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.86 (s, 3H), 3.89 (s, 3H), 6.91 (m, 2H), 7.22 (d, *J*=5.2 Hz, 1H), 7.84 (m, 2H), 7.96 (s, 1H), 8.02 (m, 2H). MS (ESI): $[M+1]^+$=391.2.

5.5. General procedure D for the preparation of compounds 7a-m. Under inert and anhydrous conditions, a 10 mL Schlenk vessel was charged with the appropriate bromothiophene derivative **5a-e** (1 mmol). The material was dissolved in a mixture of TEA-DMF (1:1, 4 mL). The corresponding acetylene derivatives (2 mmol), CuI (20 mg, 0.1 mmol) and $PdCl_2(PPh_3)_2$ (70 mg, 0.1 mmol) were then added to the solution, which was stirred at 80 °C for 16 h. The mixture was cooled to room temperature and filtered through celite. The celite was rinsed with dichloromethane, and the filtrate was concentrated *in vacuo*. The residue was fractionated between water (5 mL) and dichloromethane (15 mL). The organic layer was washed with brine (5 mL) and dried over Na_2SO_4, and the solvent was evaporated. The crude material was purified by column chromatography on silica gel.

5.5.1. Methyl 5-[(2,5-dimethoxyphenyl)ethynyl]-2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-thiophene-3-carboxylate (7a). Following general procedure D, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **7a** as a yellow solid (58% yield); mp 60-62 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.74 (s, 3H), 3.80 (s, 3H), 3.88 (s, 3H), 6.86 (s, 1H), 6.90 (d, *J*=2.8 Hz, 1H), 7.02 (d, *J*=2.8 Hz, 1H), 7.67 (s, 1H), 7.84 (m, 2H), 7.99 (m, 2H). MS (ESI): $[M+1]^+$=448.2.

5.5.2. Ethyl 5-[(2,5-dimethoxyphenyl)ethynyl]-2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-thiophene-3-carboxylate (7b). Following general procedure D, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **7b** as a yellow oil (48% yield). ^{1}H-NMR ($CDCl_3$) δ: 1.12 (t, *J*=7.0 Hz, 3H), 3.81 (s, 3H), 3.88 (s, 3H), 4.18 (q, *J*=7.0 Hz, 2H), 6.86 (s, 1H), 6.89 (d, *J*=2.8 Hz, 1H), 7.03 (d, *J*=2.8 Hz, 1H), 7.69 (s, 1H), 7.83 (m, 2H), 7.99 (m, 2H). MS (ESI): $[M+1]^+$=462.1.

5.5.3. 5-[(2,5-Dimethoxyphenyl)ethynyl]-2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-thiophene-3-carbonitrile (7c). Following general procedure D, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **7c** as a brown solid (54% yield); mp 156-158 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.80 (s, 3H), 3.88 (s, 3H), 6.95 (m, 2H), 7.01 (s, 1H), 7.40 (s, 1H), 7.89 (m, 2H), 8.03 (m, 2H). MS (ESI): $[M+1]^+$=415.2.

5.5.4. Methyl 5-[(2,5-dimethoxyphenyl)ethynyl]-2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-4-methylthiophene-3-carboxylate (7d). Following general procedure D, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **7d** as an orange solid (52% yield); mp 63-65 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.60 (s, 3H), 3.70 (s, 3H), 3.80 (s, 3H), 3.88 (s, 3H), 6.88 (m, 2H), 7.02 (s, 1H), 7.83 (m, 2H), 7.99 (m, 2H). MS (ESI): $[M+1]^+$=462.2.

5.5.5. Diethyl 5-[(2,5-dimethoxyphenyl)ethynyl]-2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)thiophene-3,4-dicarboxylate (7e). Following general procedure D, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **7e** as a yellow solid (61% yield), mp 91-92 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.14 (t, *J*=7.2 Hz, 3H), 1.39 (t, *J*=7.2 Hz, 3H), 3.80 (s, 3H), 3.87 (s, 3H), 4.19 (q, *J*=7.2 Hz, 2H), 4.40 (q,

J=7.2 Hz, 2H), 6.88 (m, 2H), 7.00 (d, *J*=2.6 Hz, 1H), 7.83 (m, 2H), 7.98 (m, 2H). MS (ESI): $[M+1]^+$=554.4.

5.5.6. Methyl 2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-5-[(2-methoxyphenyl) ethynyl]thiophene-3-carboxylate (7f). Following general procedure D, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **7f** as a yellow solid (59% yield); mp 140-141 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.73 (s, 3H), 3.91 (s, 3H), 6.92 (m, 2H), 7.35 (m, 1H), 7.47 (dd, *J* =7.6 and 1.8 Hz, 1H), 7.65 (s, 1H), 7.81 (m, 2H), 7.98 (m, 2H). MS (ESI): $[M+1]^+$=418.2.

5.5.7. Methyl 2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-5-[(4-methoxyphenyl) ethynyl]thiophene-3-carboxylate (7g). Following general procedure D, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **7g** as a yellow solid (52% yield); mp 170-172 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.73 (s, 3H), 3.84 (s, 3H), 6.89 (d, *J*=8.8 Hz, 2H), 7.46 (d, *J*=8.8 Hz, 2H), 7.60 (s, 1H), 7.82 (m, 2H), 7.98 (m, 2H). MS (ESI): $[M+1]^+$=418.1.

5.5.8. Methyl 2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-5-[(4-methoxy-2-methylphenyl) ethynyl]thiophene-3-carboxylate (7h). Following general procedure D, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **7h** as a brown solid (51% yield); mp 158-160 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.46 (s, 3H), 3.73 (s, 3H), 3.82 (s, 3H), 6.75 (m, 2H), 7.39 (m, 1H), 7.59 (s, 1H), 7.83 (m, 2H), 7.98 (m, 2H). MS (ESI): $[M+1]^+$=432.1.

5.5.9. Ethyl 2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-5-[(2-methoxyphenyl) ethynyl]thiophene-3-carboxylate (7i). Following general procedure D, the crude residue

purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **7i** as a brown oil (53% yield). ^{1}H-NMR ($CDCl_3$) δ: 1.11 (t, *J*=7.0 Hz, 3H), 3.91 (s, 3H), 4.16 (q, *J*=7.0 Hz, 2H), 6.92 (m, 2H), 7.35 (m, 1H), 7.46 (m, 1H), 7.67 (s, 1H), 7.82 (m, 2H), 7.98 (m, 2H). MS (ESI): $[M+1]^+$=432.2.

5.5.10. Methyl 2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-5-[(4-methoxyphenyl) ethynyl]thiophene-3-carboxylate (7j). Following general procedure D, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **7j** as a yellow solid (56% yield); mp 139-140 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.06 (t, *J*=7.2 Hz, 3H), 3.84 (s, 3H), 4.16 (q, *J*=7.2 Hz, 2H), 6.89 (d, *J*=8.8 Hz, 2H), 7.46 (d, *J*=8.8 Hz, 2H), 7.62 (s, 1H), 7.84 (m, 2H), 7.96 (m, 2H). MS (ESI): $[M+1]^+$=432.1.

5.5.11. 2-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-5-[(2-methoxyphenyl) ethynyl]thiophene-3-carbonitrile (7k). Following general procedure D, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **7k** as a brown solid (54% yield); mp 139-140 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.91 (s, 3H), 6.95 (m, 2H), 7.36 (m, 3H), 7.87 (m, 2H), 8.03 (m, 2H). MS (ESI): $[M+1]^+$=385.1.

5.5.12. 2-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-5-[(4-methoxyphenyl) ethynyl]thiophene-3-carbonitrile (7l). Following general procedure D, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **7l** as a brown solid (54% yield); mp 160-162 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.82 (s, 3H), 6.88 (d, *J*=8.8 Hz, 2H), 6.95 (m, 2H), 7.31 (s, 1H), 7.37 (d, *J*=8.8 Hz, 2H), 7.87 (m, 2H), 7.99 (m, 2H). MS (ESI): $[M+1]^+$=385.1.

5.5.13. 2-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-5-(phenylethynyl) thiophene-3-carbonitrile (7m). Following general procedure D, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **7m** as a brown solid (43% yield); mp 169-171 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.39 (m, 4H), 7.52 (m, 2H), 7.87 (m, 2H), 8.03 (m, 2H). MS (ESI): $[M+1]^+$=355.2.

5.6. General procedure E for the synthesis of compounds 8a-m and 2n-o. A stirred suspension of a thiophene derivative **7a-m** and **6a-b** (1 mmol) and hydrazine monohydrate (60 μL, 1.2 mmol, 1.2 equiv.) in abs. EtOH (10 mL) was refluxed for 1 h. The solvent was evaporated, and the residue was partitioned between dichloromethane (20 mL) and water (5 mL). The separated organic phase, washed with brine (5 mL) and dried over Na_2SO_4, was concentrated under reduced pressure to obtain a residue that was purified by column chromatography.

5.6.1. Methyl 2-amino-5-[(2,5-dimethoxyphenyl)ethynyl]thiophene-3-carboxylate (8a). Following general procedure E, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **8a** as a yellow solid (90% yield); mp 147-148 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.78 (s, 3H), 3.81 (s, 3H), 3.86 (s, 3H), 6.17 (bs, 2H), 6.82 (s, 1H), 6.97 (m, 1H), 7.67 (s, 1H), 7.21 (s, 1H). MS (ESI): $[M+1]^+$=317.1.

5.6.2. Ethyl 2-amino-5-[(2,5-dimethoxyphenyl)ethynyl]thiophene-3-carboxylate (8b). Following general procedure E, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **8b** as a yellow oil (85% yield). ^{1}H-NMR ($CDCl_3$) δ: 1.35 (t, *J*=7.0 Hz, 3H), 3.78 (s, 3H), 3.86 (s, 3H), 4.29 (q, *J*=7.0 Hz, 2H), 6.13 (bs, 2H), 6.83 (d, *J*=3.4 Hz, 1H), 6.84 (s, 1H), 6.98 (d, *J*=3.4 Hz, 1H), 7.23 (s, 1H). MS (ESI): $[M+1]^+$=332.3.

5.6.3. 2-Amino-5-[(2,5-dimethoxyphenyl)ethynyl]thiophene-3-carbonitrile (8c). Following general procedure E, the crude residue purified, by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **8c** as a brown solid (73% yield), mp 122-124 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.78 (s, 3H), 3.86 (s, 3H), 4.93 (bs, 2H), 6.85 (m, 2H), 6.96 (m, 3H). MS (ESI): $[M+1]^+$=285.1.

5.6.4. Methyl 2-amino-5-[(2,5-dimethoxyphenyl)ethynyl]-4-methylthiophene-3-carboxylate (8d). Following general procedure E, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **8d** as a yellow solid (77% yield); mp 100-102 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.46 (s, 3H), 3.78 (s, 3H), 3.84 (s, 3H), 3.86 (s, 3H), 6.50 (bs, 2H), 6.83 (m, 2H), 6.98 (m, 1H). MS (ESI): $[M+1]^+$=332.1.

5.6.5. Diethyl 2-amino-5-[(2,5-dimethoxyphenyl)ethynyl]thiophene-3,4-dicarboxylate (8e). Following general procedure E, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **8e** as a yellow solid (78% yield), mp 103-104 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.32 (t, *J*=7.2 Hz, 3H), 1.38 (t, *J*=7.2 Hz, 3H), 3.78 (s, 3H), 3.84 (s, 3H), 4.26 (q, *J*=7.0 Hz, 2H), 4.38 (q, *J*=7.0 Hz, 2H), 6.23 (bs, 2H), 6.83 (m, 2H), 6.94 (d, *J*=2.0 Hz, 1H). MS (ESI): $[M+1]^+$=404.2.

5.6.6. Methyl-2-Amino-5-[(2-methoxyphenyl)ethynyl]thiophene-3-carboxylate (8f). Following general procedure E, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **8f** as a yellow oil (82% yield). ^{1}H-NMR ($CDCl_3$) δ: 3.81 (s, 3H), 3.89 (s, 3H), 6.12 (bs, 2H), 6.88 (m, 2H), 7.20 (s, 1H), 7.26 (m, 1H), 7.42 (dd, *J*=7.4 and 1.4 Hz, 1H). MS (ESI): $[M+1]^+$=288.2.

5.6.7. Methyl-2-amino-5-[(4-methoxyphenyl)ethynyl]thiophene-3-carboxylate (8g). Following general procedure E, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **8g** as a yellow solid (74% yield), mp 108-110 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.81 (s, 3H), 3.86 (s, 3H), 6.12 (bs, 2H), 6.85 (dd, *J*=8.8 and 1.8 Hz, 2H), 7.14 (s, 1H), 7.40 (dd, *J*=8.6 and 1.8 Hz, 2H). MS (ESI): $[M+1]^+$=288.2.

5.6.8. Methyl-2-amino-5-[(4-methoxy-2-methylphenyl)ethynyl]thiophene-3-carboxylate (8h). Following general procedure E, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **8h** as a brown solid (88% yield), mp 121-122 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.42 (s, 3H), 3.81 (s, 3H), 3.82 (s, 3H), 6.10 (bs, 2H), 6.73 (m, 2H), 7.13 (s, 1H), 7.35 (d, *J*=8.6 Hz, 1H). MS (ESI): $[M+1]^+$=302.2.

5.6.9. Ethyl-2-amino-5-[(2-methoxyphenyl)ethynyl]thiophene-3-carboxylate (8i). Following general procedure E, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **8i** as a brown oil (52% yield). ^{1}H-NMR ($CDCl_3$) δ: 1.33 (t, *J*=7.2 Hz, 3H), 3.89 (s, 3H), 4.26 (q, *J*=7.2 Hz, 2H), 6.15 (bs, 2H), 6.90 (m, 2H), 7.22 (s, 1H), 7.31 (m, 2H). MS (ESI): $[M+1]^+$=302.1.

5.6.10. Ethyl-2-amino-5-[(4-methoxyphenyl)ethynyl]thiophene-3-carboxylate (8j). Following general procedure E, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **8j** as a yellow solid (89% yield), mp 90-92 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.33 (t, *J*=7.2 Hz, 3H), 3.81 (s, 3H), 4.26 (q, *J*=7.2 Hz, 2H), 6.14 (bs, 2H), 6.84 (d, *J*=8.8 Hz, 2H), 7.16 (s, 1H), 7.36 (d, *J*=8.6 Hz, 2H). MS (ESI): $[M+1]^+$=302.0.

5.6.11. 2-Amino-5-[(2-methoxyphenyl)ethynyl]thiophene-3-carbonitrile (8k). Following general procedure E, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **8k** as a brown oil (74% yield). ^{1}H-NMR ($CDCl_3$) δ: 3.89 (s, 3H), 4.93 (bs, 2H), 6.92 (m, 2H), 7.30 (m, 2H), 7.42 (dd, *J*=7.4 and 1.6 Hz, 1H). MS (ESI): $[M+1]^+$=255.1.

5.6.12. 2-Amino-5-[(4-methoxyphenyl)ethynyl]thiophene-3-carbonitrile (8l). Following general procedure E, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **8l** as a brown solid (73% yield), mp 185-186 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.83 (s, 3H), 4.92 (bs, 2H), 6.87 (m, 3H), 7.41 (d, *J*=9.2 Hz, 2H). MS (ESI): $[M+1]^+$=255.0.

5.6.13. 2-Amino-5-(phenylethynyl)thiophene-3-carbonitrile (8m). Following general procedure E, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **8m** as a yellow solid (82% yield), mp 173-174 °C. ^{1}H-NMR ($CDCl_3$) δ: 4.98 (bs, 2H), 6.91 (s, 1H), 7.39 (m, 5H). MS (ESI): $[M+1]^+$=225.1.

5.6.14. Methyl 2-amino-5-(2,5-dimethoxyphenyl)thiophene-3-carboxylate (2n). Following general procedure E, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **2n** as a white solid (63% yield); mp 204-205 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.78 (s, 3H), 3.81 (s, 3H), 3.84 (s, 3H), 5.89 (bs, 2H), 6.77 (dd, *J*=8.8 and 3.0 Hz, 1H), 6.90 (d, *J*=8.8 Hz, 1H), 7.09 (d, J=3.0 Hz, 1H), 7.39 (s, 1H). MS (ESI): $[M+1]^+$=294.1. Anal. ($C_{14}H_{15}NO_4S$) C, H, N.

5.6.15. 2-Amino-5-(2,5-dimethoxyphenyl)thiophene-3-carbonitrile (2o). Following general procedure E, the crude residue purified by flash chromatography using ethyl acetate:petroleum

ether 3:7 (v:v) for elution, furnished **2o** as a cream-colored solid (74% yield), mp 126-127 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.81 (s, 3H), 3.86 (s, 3H), 6.56 (bs, 2H), 6.78 (dd, *J*=8.8 and 3.0 Hz, 1H), 6.84 (d, *J*=8.8 Hz, 1H), 7.11 (m, 2H). MS (ESI): $[M+1]^+$=260.3. Anal. ($C_{13}H_{12}N_2O_2S$) C, H, N.

5.7. General procedure F for the synthesis of compounds 2a-m. A solution of phenylalkynyl derivative **9a-m** (0.5 mmol) in EtOH (10 mL) was hydrogenated over 50 mg of 10% Pd/C at 50 p.s.i for 4 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to furnish a residue that was purified by column chromatography on silica gel.

5.7.1. Methyl 2-amino-5-[2-(2,5-dimethoxyphenyl)ethyl]thiophene-3-carboxylate (2a). Following general procedure F, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **2a** as a yellow solid (77% yield); mp 107-108 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.86 (s, 4H), 3.75 (s, 3H), 3.79 (s, 6H), 5.80 (bs, 2H), 6.71 (m, 4H). MS (ESI): $[M+1]^+$=322.2. Anal. ($C_{16}H_{19}NO_4S$) C, H, N.

5.7.2. Ethyl 2-amino-5-[2-(2,5-dimethoxyphenyl)ethyl]thiophene-3-carboxylate (2b). Following general procedure F, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **2b** as a yellow oil (86% yield). ^{1}H-NMR ($CDCl_3$) δ: 1.34 (t, *J*=7.2 Hz, 3H), 2.86 (s, 4H), 3.75 (s, 3H), 3.79 (s, 3H), 4.26 (q, *J*=7.2 Hz, 2H), 5.79 (bs, 2H), 6.72 (m, 4H). MS (ESI): $[M+1]^+$=336.1. Anal. ($C_{17}H_{21}NO_4S$) C, H, N.

5.7.3. 2-Amino-5-[2-(2,5-dimethoxyphenyl)ethyl]thiophene-3-carbonitrile (2c). Following general procedure F, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **2c** as a yellow solid (70% yield), mp

89-91 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.86 (s, 4H), 3.77 (s, 6H), 4.62 (bs, 2H), 6.37 (s, 1H), 6.72 (m, 3H). MS (ESI): $[M+1]^+$=289.1. Anal. ($C_{15}H_{16}N_2O_2S$) C, H, N.

5.7.4. Methyl 2-amino-5-[2-(2,5-dimethoxyphenyl)ethyl]-4-methylthiophene-3-carboxylate (2d). Following general procedure F, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **2d** as a yellow oil (82% yield). ^{1}H-NMR ($CDCl_3$) δ: 2.12 (s, 3H), 2.79 (s, 4H), 3.77 (s, 3H), 3.80 (s, 3H), 3.81 (s, 3H), 5.93 (bs, 2H), 6.72 (m, 3H). MS (ESI): $[M+1]^+$=336.2. Anal. ($C_{17}H_{21}NO_4S$) C, H, N.

5.7.5. Diethyl 2-amino-5-[2-(2,5-dimethoxyphenyl)ethyl]thiophene-3,4-dicarboxylate (2e). Following general procedure F, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **2b** as a yellow oil (81% yield). ^{1}H-NMR ($CDCl_3$) δ: 1.24 (t, *J*=7.2 Hz, 3H), 1.34 (t, *J*=7.2 Hz, 3H), 2.86 (s, 4H), 3.74 (s, 6H), 4.22 (q, *J*=7.2 Hz, 2H), 4.26 (q, *J*=7.2 Hz, 2H), 6.02 (bs, 2H), 6.71 (m, 3H). MS (ESI): $[M+1]^+$=408.3. Anal. ($C_{20}H_{25}NO_6S$) C, H, N.

5.7.6. Methyl 2-amino-5-[2-(2-methoxyphenyl)ethyl]thiophene-3-carboxylate (2f). Following general procedure F, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **2f** as a yellow oil (87% yield). ^{1}H-NMR ($CDCl_3$) δ: 2.87 (s, 4H), 3.78 (s, 3H), 3.82 (s, 3H), 5.83 (bs, 2H), 6.66 (s, 1H), 6.88 (m, 2H), 7.15 (m, 2H). MS (ESI): $[M+1]^+$=291.1. Anal. ($C_{15}H_{17}NO_3S$) C, H, N.

5.7.7. Methyl 2-amino-5-[2-(4-methoxyphenyl)ethyl]thiophene-3-carboxylate (2g). Following general procedure F, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **2g** as a colorless oil (>95%

yield). ^{1}H-NMR ($CDCl_3$) δ: 2.87 (s, 4H), 3.78 (s, 3H), 3.83 (s, 3H), 5.81 (bs, 2H), 6.62 (s, 1H), 6.82 (d, *J*=8.6 Hz, 2H), 7.09 (d, *J*=8.6 Hz, 2H). MS (ESI): $[M+1]^+$=291.1. Anal. ($C_{15}H_{17}NO_3S$) C, H, N.

5.7.8. Methyl 2-amino-5-[2-(4-methoxy-2-methylphenyl)ethyl]thiophene-3-carboxylate (2h). Following general procedure F, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **2h** as a yellow solid (71% yield), mp 110-112 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.30 (s, 3H), 2.82 (s, 4H), 3.81 (s, 6H), 5.85 (bs, 2H), 6.70 (m, 3H), 7.06 (d, *J*=8.0 Hz, 1H). MS (ESI): $[M+1]^+$=306.2. Anal. ($C_{16}H_{19}NO_3S$) C, H, N.

5.7.9. Ethyl 2-amino-5-[2-(2-methoxyphenyl)ethyl]thiophene-3-carboxylate (2i). Following general procedure F, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **2i** as a colorless oil (>95% yield). ^{1}H-NMR ($CDCl_3$) δ: 1.33 (t, *J*=7.0 Hz, 3H), 2.87 (s, 4H), 3.83 (s, 3H), 4.25 (q, *J*=7.0 Hz, 2H), 5.80 (bs, 2H), 6.66 (s, 1H), 6.67 (s, 1H), 6.87 (m, 2H), 7.25 (m, 1H). MS (ESI): $[M+1]^+$=306.2. Anal. ($C_{16}H_{19}NO_3S$) C, H, N.

5.7.10. Ethyl 2-amino-5-[2-(4-methoxyphenyl)ethyl]thiophene-3-carboxylate (2j). Following general procedure F, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 2:8 (v:v) for elution, furnished **2j** as a colorless oil (>95% yield). ^{1}H-NMR ($CDCl_3$) δ: 1.33 (t, *J*=7.2 Hz, 3H), 2.84 (s, 4H), 3.79 (s, 3H), 4.23 (q, *J* = 7.2 Hz, 2H), 5.81 (bs, 2H), 7.64 (s, 1H), 6.82 (d, *J*=8.6 Hz, 2H), 7.10 (d, *J*=8.6 Hz, 2H). MS (ESI): $[M+1]^+$=306.2. Anal. ($C_{16}H_{19}NO_3S$) C, H, N.

5.7.11. 2-Amino-5-[2-(2-methoxyphenyl)ethyl]thiophene-3-carbonitrile (2k). Following general procedure F, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **2k** as a brown oil (52% yield). ^{1}H-NMR ($CDCl_3$) δ: 2.88 (s, 4H), 3.84 (s, 3H), 4.64 (bs, 2H), 6.36 (s, 1H), 6.89 (m, 2H), 7.17 (m, 3H). MS (ESI): $[M+1]^+$=259.2. Anal. ($C_{14}H_{14}N_2OS$) C, H, N.

5.7.12. 2-Amino-5-[2-(4-methoxyphenyl)ethyl]thiophene-3-carbonitrile (2l). Following general procedure F, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **2l** as a brown solid (70% yield), mp 145-147 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.85 (s, 4H), 3.80 (s, 3H), 4.62 (bs, 2H), 6.34 (s, 1H), 6.83 (d, *J*=8.8 Hz, 2H), 7.08 (d, *J*=8.8 Hz, 2H). MS (ESI): $[M+1]^+$=259.3. Anal. ($C_{14}H_{14}N_2OS$) C, H, N.

5.7.13. 2-Amino-5-phenylethylthiophene-3-carbonitrile (2m). Following general procedure F, the crude residue purified by flash chromatography using ethyl acetate:petroleum ether 3:7 (v:v) for elution, furnished **2m** as an orange solid (65% yield), mp 127-129 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.90 (s, 4H), 4.63 (bs, 2H), 6.35 (s, 1H), 7.23 (m, 5H). MS (ESI): $[M+1]^+$=229.1. Anal. ($C_{13}H_{12}N_2S$) C, H, N.

5.8. Biology Experiments

5.8.1. Antiproliferative assays. Cancer cells were suspended at 300,000-500,000 cells/mL of culture medium, and 100 µL of a cell suspension was added to 100 µL of an appropriate dilution of the test compounds in wells of 96-well microtiter plates. After incubation at 37 °C for 48 h, cell number was determined using a Coulter counter. The IC_{50} was defined as the compound concentration required to inhibit cell proliferation by 50%.

5.8.2. Effects on tubulin polymerization and on colchicine binding to tubulin. To evaluate the effect of the compounds on tubulin assembly in vitro,[14] varying concentrations of compounds were preincubated with 10 μM bovine brain tubulin in glutamate buffer at 30 ˚C and then cooled to 0 ˚C. After addition of 0.4 mM GTP, the mixtures were transferred to 0 °C cuvettes in a recording spectrophotometer and warmed to 30 °C. Tubulin assembly was followed turbidimetrically at 350 nm. The IC_{50} was defined as the compound concentration that inhibited the extent of assembly by 50% after a 20 min incubation. The capacity of the test compounds to inhibit colchicine binding to tubulin was measured as described,[16] except that the reaction mixtures contained 1 μM tubulin, 5 μM [^{3}H]colchicine and 5 μM test compound.

5.8.3. Molecular Modeling. All molecular modeling studies were performed on a MacPro dual 2.66GHz Xeon running Ubuntu 10. The tubulin structure was downloaded from the PDB data bank (http://www.rcsb.org/ - PDB code: 3HKC).[30] The 3HKC structure was chosen based on the structural similarities of the co-crystallized ligand present in this complex over the ligands present in other tubulin structures available.[17] Hydrogen atoms were added to the protein, using the Protonate3D function of Molecular Operating Environment (MOE).[31] Ligand structures were built with MOE and minimized using the MMFF94x forcefield until a RMSD gradient of 0.05 kcal mol^{-1} Å^{-1} was reached. The docking simulations were performed using PLANTS.[32] Molecular dynamics was performed with the Gromacs 4.5[33] with the Amber99 force field. The structure was solvated using TIP3P water molecules, providing a minimum of 9.0 Å of water between the protein surface and any periodic box edge. The system was neutralized, minimized and then a position restrain dynamics simulation was carried out for 150 ps. The production simulation was conducted for 3 ns at 298 K using a

NPT environment. Inhibitors were parameterized by Antechamber of AmberTool 1.5.[34] Trajectories analysis were carried out by VMD.[35]

5.8.4. Flow cytometric analysis of cell cycle distribution. For flow cytometric analysis of DNA content, $5x10^5$ HeLa cells in exponential growth were treated with different concentrations of the test compounds for 24 or 48 h. After the incubation period, the cells were collected, centrifuged and fixed with ice-cold ethanol (70%). The cells were then treated with lysis buffer containing RNAse A and 0.1% Triton X-100, and then stained with PI. Samples were analyzed on a Cytomic FC500 flow cytometer (Beckman Coulter). DNA histograms were analyzed using MultiCycle® for Windows (Phoenix Flow Systems).

5.8.5. Annexin-V assay. Surface exposure of PS on apoptotic cells was measured by flow cytometry with a Coulter Cytomics FC500 (Beckman Coulter) by adding annexin-V-FITC to cells according to the manufacturer's instructions (Annexin-V Fluos, Roche Diagnostic). Simultaneously, the cells were stained with PI. Excitation was set at 488 nm, and the emission filters were at 525 and 585 nm, respectively, for FTIC and PI.

5.8.6. Assessment of mitochondrial changes. The mitochondrial membrane potential was measured with the lipophilic cationic dye JC-1 (Molecular Probes), as described.[24] The production of ROS was measured by flow cytometry using either HE (Molecular Probes) or H_2DCFDA (Molecular Probes), as previously described.[24]

5.8.7. Western Blot Analysis. HeLa cells were incubated in the presence of test compounds and, after different times, were collected, centrifuged and washed two times with ice cold phosphate-buffered saline (PBS). The pellet was then resuspended in lysis buffer containing T-PER Reagent (Pierce, Milano Italy), sodium chloride (300 mM), sodium orthovanadate (1 mM), AEBSF, (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (2 mM), Aprotinin

(1 µg/ml), Pepstatin A (5 µg/ml), Leupeptin (1 µg/ml). After the cells were lysed on ice for 30 min, lysates were centrifuged at 15000 x g at 4 °C for 10 min. The protein concentration in the supernatant was determined using the BCA protein assay reagents (Pierce, Italy). Equal amounts of protein (20 µg) were resolved using SDS-PAGE (7.5-15 % acrylamide gels) and transferred to PVDF Hybond-p membrane (GE Healthcare). Membranes were blocked with I-block (Tropix), the membrane being gently rotated overnight at 4 °C. Membranes were then incubated with primary antibodies against Bcl-2, Mcl-1, Xiap, PARP, cleaved caspase-9, p-cdc2^{Tyr15}, cdc25c (Cell Signaling), caspase-3 (Alexis), cyclin B (Upstate) or β-actin (Sigma-Aldrich) for 2 h at room temperature. Membranes were next incubated with peroxidase-labeled secondary antibodies for 60 min. All membranes were visualized using ECL Advance (GE Healthcare) and exposed to Hyperfilm MP (GE Healthcare). To ensure equal protein loading, each membrane was stripped and reprobed with anti-β-actin antibody.

5.8.8. Statistical Analysis. Unless indicated otherwise, results are presented as mean ± S.E.M. The differences between different treatments were analyzed using the two-sided Student's t test. P values less than 0.05 were considered significant.

Supplementary data available. Molecular modeling studies (**Figures 1-4s**). Elemental analyses of compounds **2a-o.** Supplementary data associated with this article can be found in the online version.

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Abbreviations. $Pd(PPh_3)_4$, tetrakis (triphenylphosphine)palladium; K_2CO_3, potassium carbonate; $PdCl_2(PPh_3)_2$, bis(triphenylphosphine)-palladium chloride; CuI, cuprous iodide;

TEA, triethylamine; DMF, N, *N'*-dimethylformamide; 10% Pd/C, 10% palladium on charcoal; SAR, structure-activity relationships; PI, propidium iodide; PS, phospholipid phosphatidylserine; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine; ROS, reactive oxygen species; HE, hydroxyethidine; H_2DCFDA, 2,7-dichlorodihydrofluorescein; PARP, poly-ADP-ribose polymerase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Table 1. In vitro inhibitory effects of compounds **1** and **2a-o** on proliferation of murine leukemia (L1210), murine mammary carcinoma (FM3A/0), human cervix carcinoma (HeLa) and human T-leukemia (Molt4/C8 and CEM) cells

Compound	IC_{50}±S.D (μM)[a]				
	L1210	FM3A/0	Molt4/C8	CEM/0	HeLa
2a	1.8±0.1	2.3±0.6	0.42±0.11	1.4±0.2	0.77±0.17
2b	1.2±0.4	1.9±0.5	1.1±0.1	0.40±0.15	0.31±0.00
2c	0.064±0.005	0.056±0.004	0.058±0.006	0.035±0.001	0.13±0.06
2d	46±3.2	44±8.1	47±16	30±3.2	35±7.1
2e	34±3.2	33±9.1	24±2.2	20±6.3	11±3.5
2f	17±5.3	34±5.6	0.81±0.09	1.2±0.1	15±1.3
2g	11±1.2	67±26	0.27±0.08	0.62±0.04	36±6.4
2h	248±40	45±16	0.27±0.27	1.3±0.1	105±68
2i	40±0.2	53±7.2	6.9±0.8	20±4.3	45±6.2
2j	20±10	48±5.2	0.34±0.50	1.9±0.9	39±0.2
2k	15±4.1	22±3.1	2.0±0.2	6.8±0.2	7.8±2.1
2l	14±4.2	31±27	1.4±0.8	7.2±4.2	19±13
2m	40±6.1	62±3.2	8.5±3.4	32±7.1	35±3.1
2n	50±5.2	52±1.0	28±6.2	30±2.2	37±6.1
2o	76±3.2	n.d.	n.d.	12±3.1	71±20
1	0.36±0.04	13±0.2	n.d.	0.44±0.01	0.41±0.08

[a]IC_{50}= compound concentration required to inhibit tumor cell proliferation by 50%. Data are expressed as the IC_{50}±SD from the dose-response curves of at least three independent experiments.
n.d.= not determined.

Table 2. Effect of compound **2c** on growth of different human cancer cell lines

Cell type	Cell line	IC_{50}±S.D (μM)[a]
T-cell lymphoma	Sup-T1	0.017±0.012
T-Leukemia	MT2	0.027±0.001
T-Leukemia	C8166	0.021±0.009
T-Leukemia	Jurkat	0.012±0.008
T-cell lymphoma	HUT78	0.062±0.019
T-Leukemia	MT4	0.086±0.048
Non-Hodgkin leukemia/B-cell lymphoma	Raji	0.064±0.025
Osteosarcoma	HOS, OST TK^-	0.024±0.013
Mammary carcinoma	MCF-7	0.025±0.001
Colon carcinoma	HT29	0.046±0.012
Glioma	U87	0.038±0.008
Prostate	DU145	0.061±0.016
Prostate	PC-3	0.025±0.013

[a]IC_{50}= compound concentration required to inhibit tumor cell proliferation by 50%. Data are expressed as the IC_{50}±SD from the dose-response curves of at least three independent experiments.

Table 3. Inhibition of tubulin polymerization and colchicine binding by compounds **1**, **2a-c**, **2f-h** and **2j**.

Compound	Tubulin assembly[a] IC_{50}±S.D (μM)	Colchicine binding [b] % ±S.D
2a	1.9±0.02	52±0.9
2b	3.1±0.2	46±0.9
2c	0.74±0.06	84±5
2f	>20	n.d.
2g	10±0.7	n.d.
2h	>20	n.d.
2j	16±1	n.d.
1	2.0±0.06	52±4

[a] Inhibition of tubulin polymerization. Tubulin was at 10 μM.
[b] Inhibition of [^{3}H]colchicine binding. Tubulin, colchicine and tested compound were at 1, 5 and 5 μM, respectively.
[a,b] Values are mean±S.D of four separate experiments.
n.d.=not determined.

Figure Legends.

Figure 1. Docking pose on β-tubulin of **2a**, **2c** and **2f**, with carbon atoms indicated in green, cyan, and brown, respectively. The co-crystallised ligand is represented with carbon atom colored in purple. Amino acid carbons are in grey, with the important βCys241 and αThr179 bolder than the other residues. Colors of other atoms: sulphur, yellow; nitrogen, blue; oxygen, red. Hydrogen atoms are not shown.

Figure 2. Binding pose on β-tubulin of **2c** after MD simulation. Colors of all atoms are as in Figure 1.

Figure 3. (A and B). Effect of compound **2c** on cell cycle distribution of HeLa (A) and Jurkat cells (B). Cells were treated with different concentrations of **2c** ranging from 15 to 250 nM for 24 h. Then the cells were fixed and stained with PI to analyze DNA content by flow cytometry. Data are presented as mean ± SEM of three independent experiments. (C). Effect of **2c** on G2/M regulatory proteins. HeLa cells were treated for 24 or 48 h with the indicated concentration of **2c**. The cells were harvested and lysed for the detection of cyclin B, p-cdc2^{Y15} and cdc25C expression by western blot analysis. To confirm equal protein loading, each membrane was stripped and reprobed with anti-β-actin antibody.

Figure 4. (A). Representative dot plots of HeLa cells treated with **2c** (62 nM) for 24 or 48 h and analyzed by flow cytometry after double staining of the cells with annexin-V-FITC and PI. (B and C). Percentage of cells found in the different regions of the biparametric histograms obtained from cytofluorimetric analysis, after incubation with **2c** for 24 (Panel B) or 48 h (Panel C), at the indicated concentrations.

Figure 5. Assessment of mitochondrial dysfunction after treatment of HeLa cells with compound **2c**. (A). Induction of loss of mitochondrial membrane potential ($\Delta\psi_{mt}$) after 24 or 48 h incubations with compound **2c** at the indicated concentrations. (B and C) Mitochondrial production of ROS in HeLa cells following treatment with compound **2c**. After the 24 or 48 h incubations, cells were stained with H_2-DCFDA (B) or HE (C). Data are expressed as mean ± S.E.M. for three independent experiments.

Figure 6. (A). Western blot analysis of cleaved caspase-3, cleaved caspase-9 and poly (ADP-ribose) polymerase (PARP) after treatment of HeLa cells with **2c** at the indicated concentrations and for the indicated times. (B). Western blot analysis of Bcl-2, Mcl-1 and XIAP after treatment of HeLa cells with **2c** at the indicated concentrations and for the indicated times. To confirm equal protein loading, each membrane was stripped and reprobed with anti-β-actin antibody.

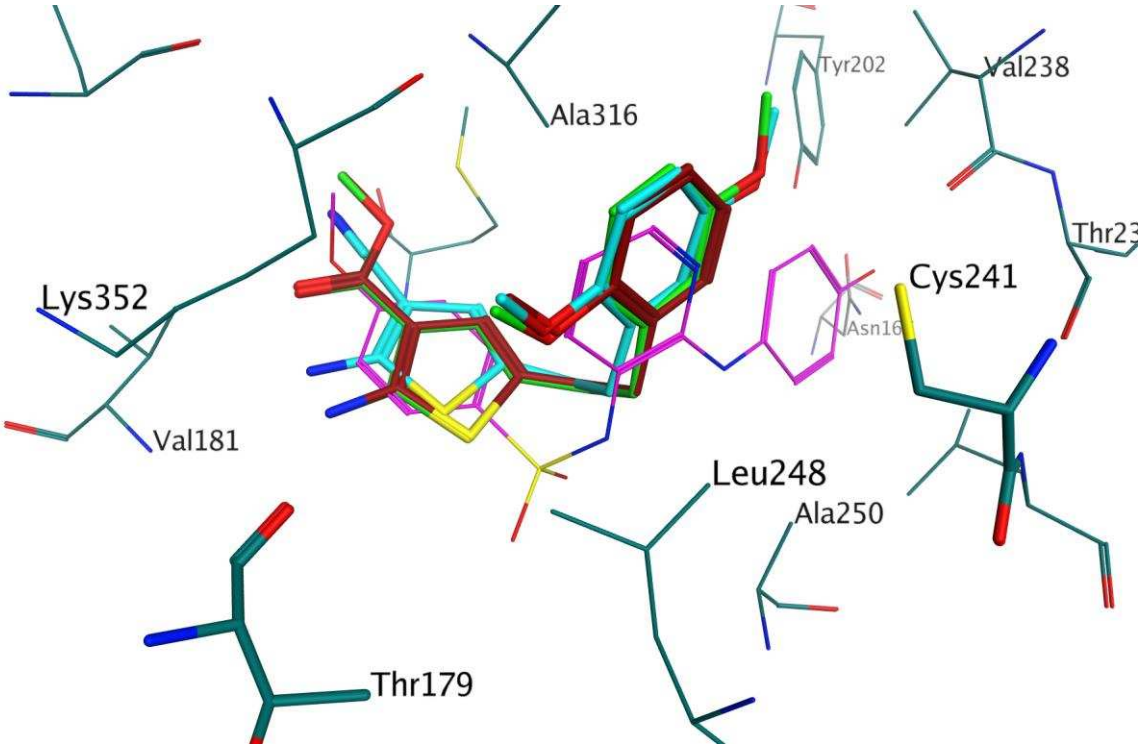

Tyr202
Val238
Ala316
Thr23
Lys352
Cys241
Asn16
Val181
Leu248
Ala250
Thr179

Figure 1.

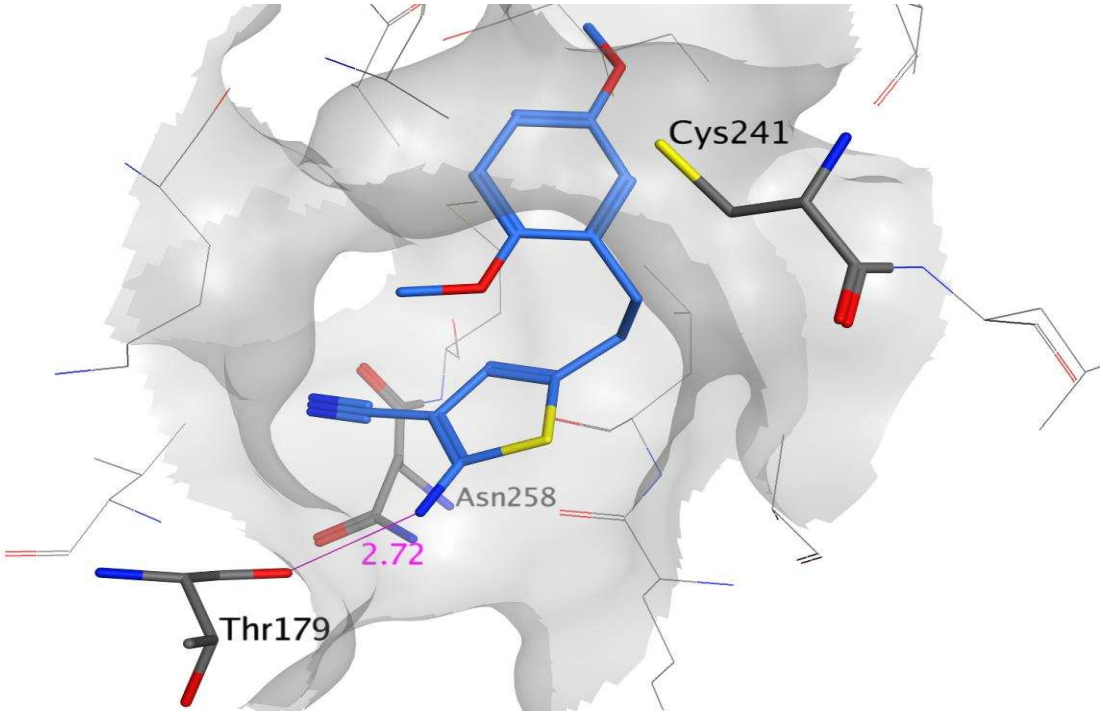


Figure 2.

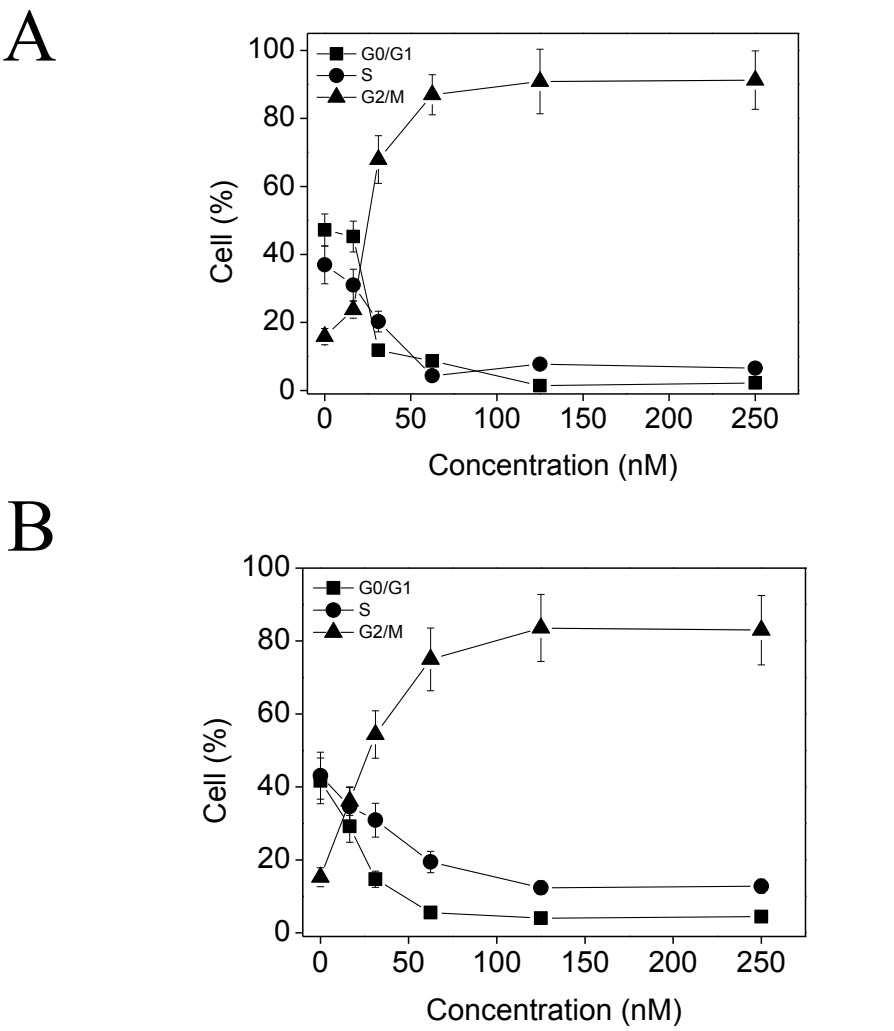
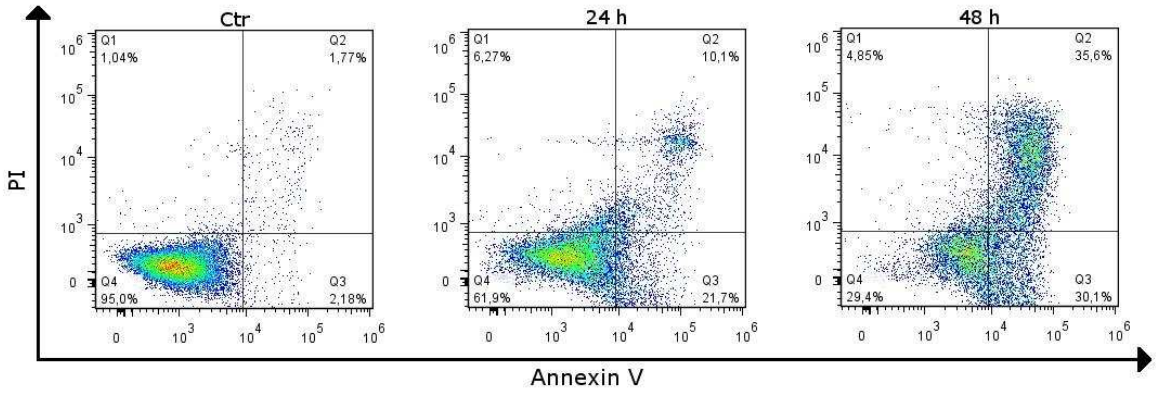
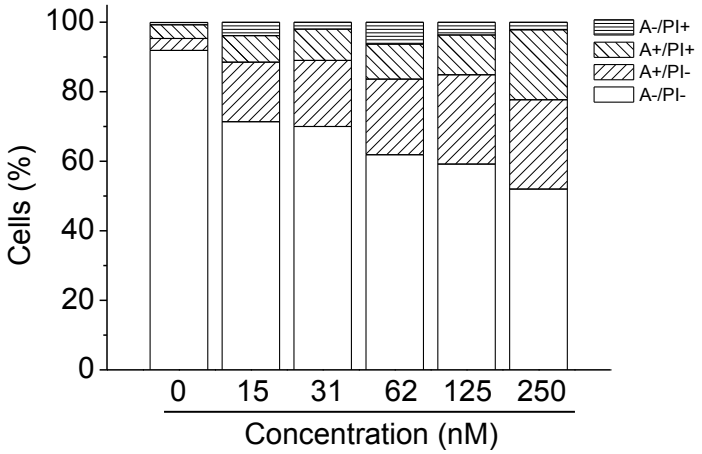

A
G0/G1
S
G2/M
Cell (%)
Concentration (nM)
B
G0/G1
S
G2/M
Cell (%)
Concentration (nM)
24 h
48 h
ctr 50 100 ctr 50 100 nM
C
- p-cdc25C
- cdc25C
- cdc2
- cyclin B
- Actin

Figure 3

A


Ctr
24 h
48 h
Q1
1,04%
Q2
1,77%
Q4
95,0%
Q3
2,18%
Q1
6,27%
Q2
10,1%
Q4
61,9%
Q3
21,7%
Q1
4,85%
Q2
35,6%
Q4
29,4%
Q3
30,1%
PI
Annexin V

B


A-/PI+
A+/PI+
A+/PI-
A-/PI-
Cells (%)
0
20
40
60
80
100
0
15
31
62
125
250
Concentration (nM)

C

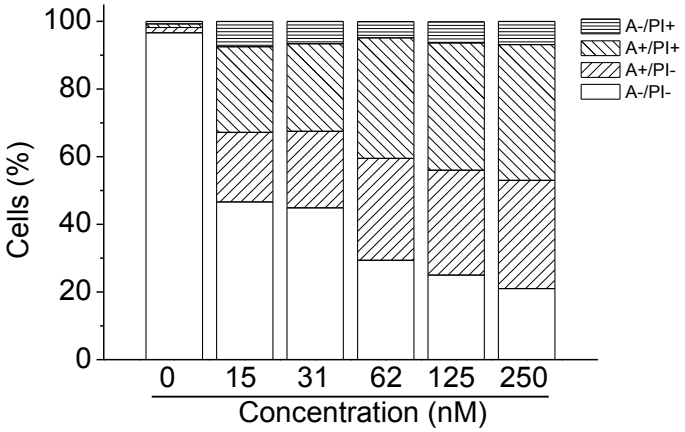

A-/PI+
A+/PI+
A+/PI-
A-/PI-
Cells (%)
0
20
40
60
80
100
0
15
31
62
125
250
Concentration (nM)

Figure 4

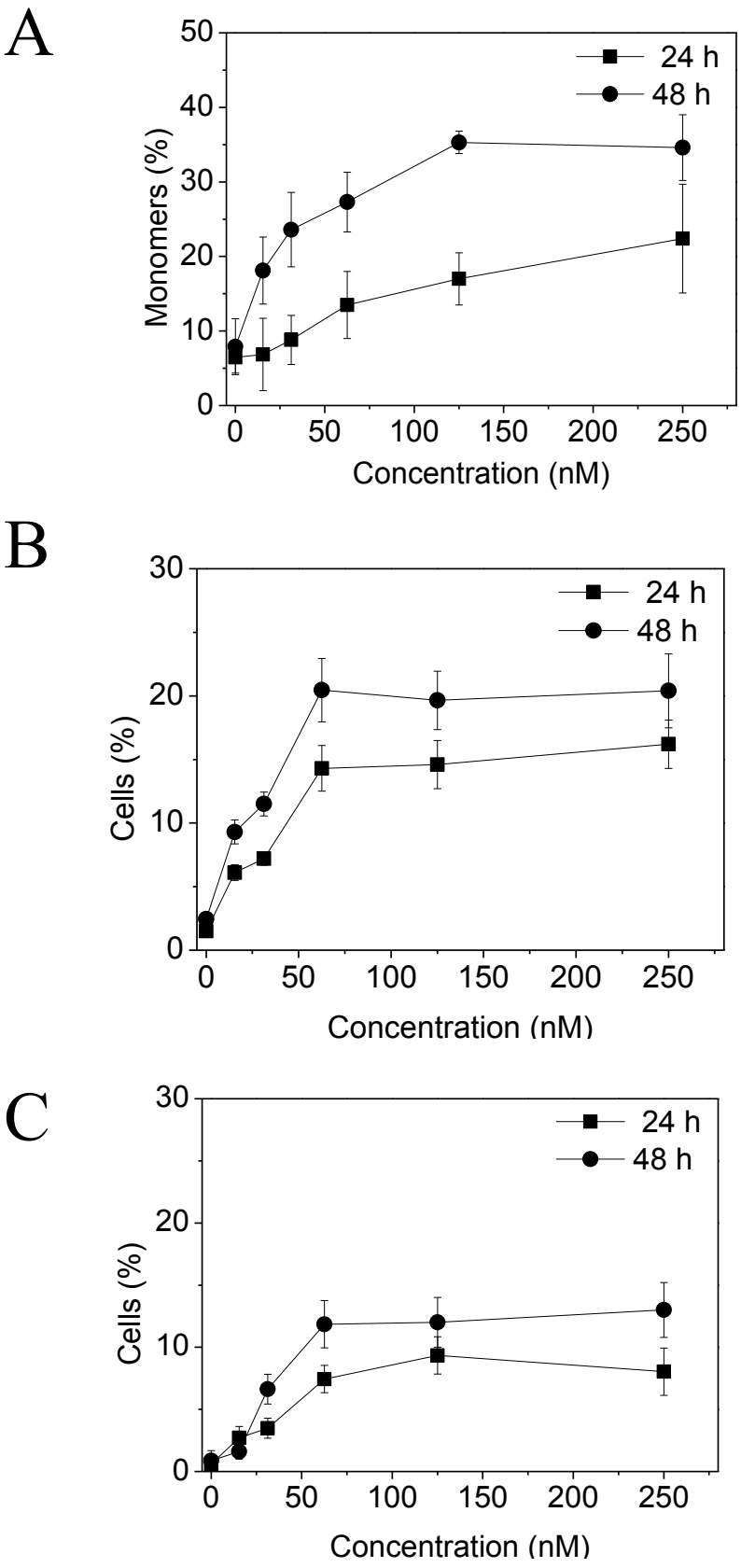

A
Monomers (%)
Concentration (nM)
24 h
48 h
B
Cells (%)
Concentration (nM)
24 h
48 h
C
Cells (%)
Concentration (nM)
24 h
48 h

Figure 5

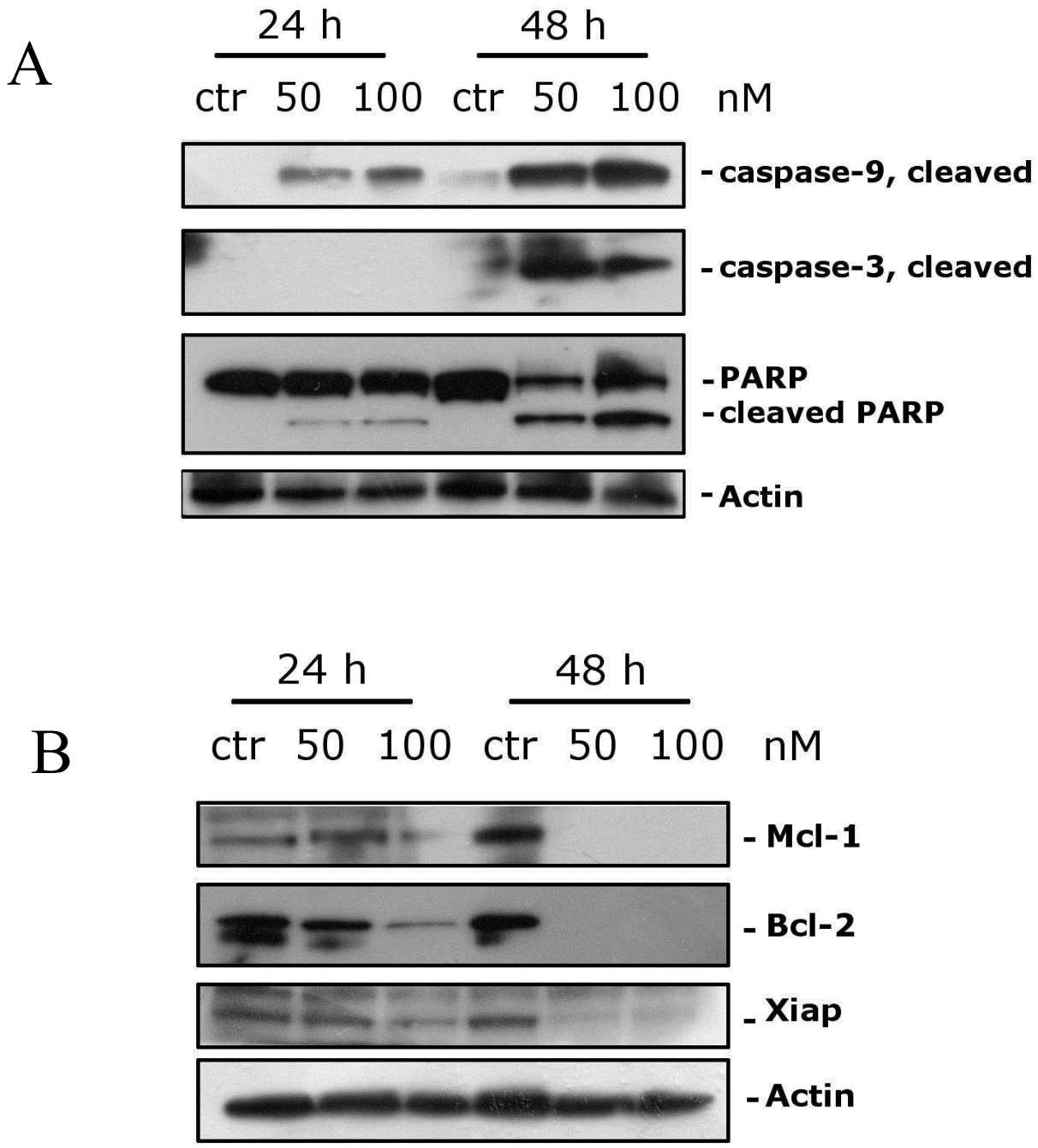
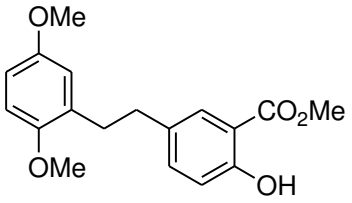
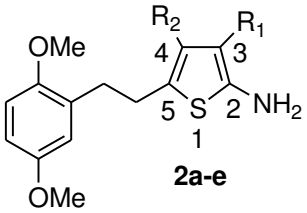

A
24 h
48 h
ctr 50 100 ctr 50 100 nM
- caspase-9, cleaved
- caspase-3, cleaved
- PARP
- cleaved PARP
- Actin
B
24 h
48 h
ctr 50 100 ctr 50 100 nM
- Mcl-1
- Bcl-2
- Xiap
- Actin

Figure 6

Chart 1. Chemical structures of SDZ LAP 977 (1) and 2-aminothiophene derivatives **2a-o**

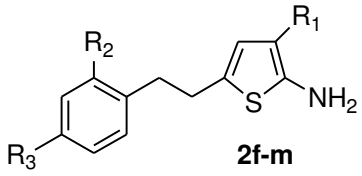


1 (SDZ LAP 977)



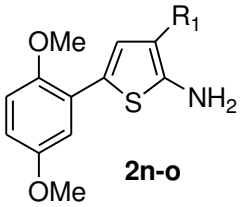
2a-e

2a, R_1=CO_2Me, R_2=H
2b, R_1=CO_2Et, R_2=H
2c, R_1=CN, R_2=H
2d, R_1=CO_2Me, R_2=Me
2e, R_1=R_2=CO_2Et



2f-m

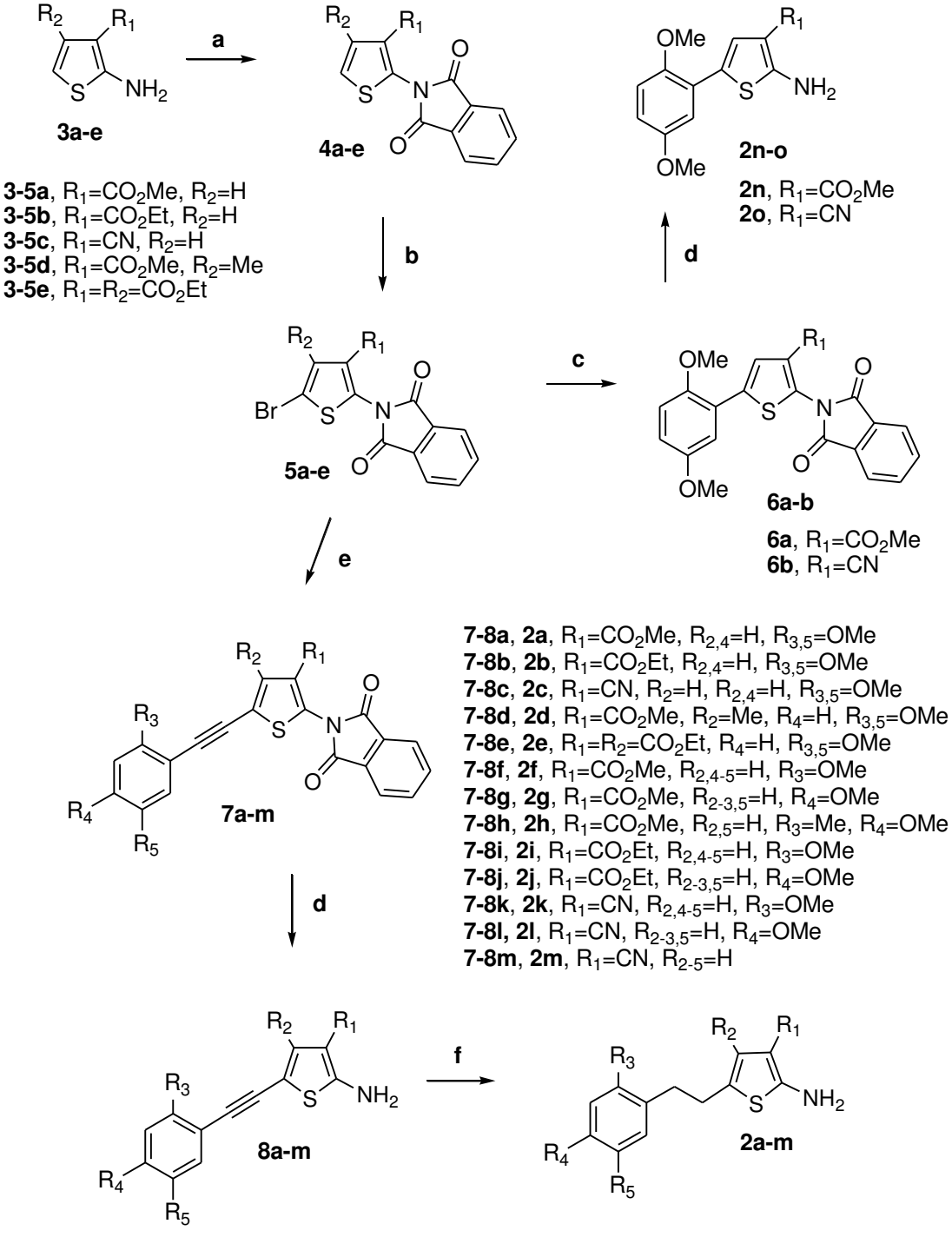
2f, R_1=CO_2Me, R_2=OMe, R_3=H
2g, R_1=CO_2Me, R_2=H, R_3=OMe
2h, R_1=CO_2Me, R_2=Me, R_3=OMe
2i, R_1=CO_2Et, R_2=OMe, R_3=H
2j, R_1=CO_2Et, R_2=H, R_3=OMe
2k, R_1=CN, R_2=OMe, R_3=H
2l, R_1=CN, R_2=H, R_3=OMe
2m, R_1=CN, R_2=R_3=H



2n-o

2n, R_1=CO_2Me
2o, R_1=CN

Scheme 1



Reagents.**a**: phthalic anhydride, AcOH; **b**: Br_2, AcOH-AcONa; **c**: 2',5'-$(OMe)_2C_6H_4B(OH)_2$, $Pd(PPh_3)_4$, K_2CO_3, $PhCH_3$, 100 °C, 18 h from **5a** and **5c**,; **d**: NH_2NH_2, EtOH; **e**. appropriate arylacetylene, $PdCl_2(PPh_3)_2$, CuI, TEA-DMF (1:1, v/v), 80 °C; **f**: H_2, 10% Pd/C, EtOH.