In Vitro Antitumor Mechanism of (E)-N-(2-methoxy-5-((2,4,6-trimethoxystyrylsulfonyl)methyl)pyridin-3-yl)methanesulfonamide\[E\]

Tiangong Lu, Charles A. Laughton, Shudong Wang, and Tracey D. Bradshaw

School of Pharmacy and Centre for Biomolecular Sciences, University of Nottingham, University Park, Nottingham, United Kingdom (T.L., C.A.L., T.D.B.); and Centre for Drug Discovery and Development, Sansom Institute for Health Research, and School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, Australia (S.W.)

Received April 16, 2014; accepted October 14, 2014

ABSTRACT

ON01910.Na [sodium (E)-2-(2-methoxy-5-((2,4,6-trimethoxystyrylsulfonyl)methyl)phenylamino)acetate; Rigosertib, Estybon], a styryl benzylsulfone, is a phase III stage anticancer agent. This non-ATP competitive kinase inhibitor has multitargeted activity, promoting mitotic arrest and apoptosis. Extensive phase I/II studies with ON01910.Na, conducted in patients with solid tumors and hematologic cancers, demonstrate excellent efficacy. However, issues remain affecting its development. These include incomplete understanding of antitumor mechanisms, low oral bioavailability, and unpredictable pharmacokinetics. We have identified a novel (E)-styrylsulfonyl methyl/pyridine [(E)-N-(2-methoxy-5-((2,4,6-trimethoxystyrylsulfonyl)methyl) pyridin-3-yl)methanesulfonamide (TL-77)] which has shown improved oral bioavailability compared with ON01910.Na. Here, we present detailed cellular mechanisms of TL-77 in comparison with ON01910.Na. TL-77 displays potent growth inhibitory activity in vitro (GI_{50} < 1 μM against HCT-116 cells), demonstrating 3- to 10-fold greater potency against tumor cell lines when compared with normal cells. Cell-cycle analyses reveal that TL-77 causes significant G2/M arrest in cancer cells, followed by the onset of apoptosis. In cell-free conditions, TL-77 potently inhibits tubulin polymerization. Mitotically arrested cells display multipolar spindles and misalignment of chromosomes, indicating that TL-77 interferes with mitotic spindle assembly in cancer cells. These effects are accompanied by induction of DNA damage, inhibition of Cdc25C phosphorylation [indicative of Plk1 inhibition], and downstream inhibition of cyclin B1. However, kinase assays failed to confirm inhibition of Plk1. Nonsignificant effects on phosphoinositide 3-kinase/Akt signal transduction were observed after TL-77 treatment. Analysis of apoptotic signaling pathways reveals that TL-77 downregulates expression of B-cell lymphoma 2 family proteins (Bid, Bcl-xl, and Mcl-1) and stimulates caspase activation. Taken together, TL-77 represents a promising anticancer agent worthy of further evaluation.

Introduction

Among the cell-cycle phases, S phase and mitosis (M) determine how cells divide and transmit genetic information from one cell generation to the next (Schmit and Ahmad, 2007). Despite being the shortest phase of the cell cycle, M orchestrates major changes in multiple cellular components (Chan et al., 2012). Mitotic kinases possess pivotal roles throughout cell division through regulating a diverse range of signaling pathways and controlling mitotic checkpoints, spindle function, and chromosome segregation. Mitotic kinases have also been implicated in tumorigenesis; aberrant cellular responses triggered by protein kinase–mediated events are thought to lead to uncontrolled proliferation, aneuploidy, and genetic instability culminating in cancer development (Schmit and Ahmad, 2007). Therefore, mitotic kinases have attracted significant attention as important targets for cancer drug discovery. The demonstration of clinical activity of a number of small-molecule inhibitors for the treatment of cancer has generated considerable interest in the search for mitotic kinase inhibitors (Smits et al., 2006; Chan et al., 2012). Non-ATP competitive inhibitors for cancer therapy are of particular interest to overcome the problems of poor selectivity and drug resistance associated with ATP analogs (Kirkland and McInnes, 2009).

ON01910.Na [sodium (E)-2-(2-methoxy-5-((2,4,6-trimethoxystyrylsulfonyl)methyl)phenylamino)acetate; also referred to as Rigosertib and Estybon] (Fig. 1), a styryl benzylsulfone, is a non-ATP competitive anticancer agent currently in phase III clinical trials. It demonstrates multitargeted activity, and induces mitotic arrest and apoptosis in cancer cells (http://www.onconova.com/product-pipeline/rogosertib.php). Initially it was thought that the antimitic activity was due to inhibition of polo-like kinase 1 (Plk1) (Gumireddy et al., 2005). However, subsequent studies did not support a direct effect on Plk1 (Steegmaier et al., 2007). Recent studies reported that ON01910.Na uses a dual molecular mechanism of action, targeting Plk1 and mitotic kinases.
inducing cell-cycle arrest correlated with RanGAP1 hyperphosphorylation and modulating the phosphoinositide 3-kinase (PI3K)/AKT pathway to promote apoptosis in cancer cells (Prasad et al., 2009; Oussenko et al., 2011). Extensive phase I/II studies with ON01910.Na have been conducted in patients with solid tumors and hematologic cancers, revealing objective durable efficacy of the drug with good tolerability (Jimeno et al., 2008; Ohnuma et al., 2013). However, a number of key issues remain that are affecting its development; these include an incomplete understanding of mechanisms of action and relatively poor drug oral bioavailability.

In a program to identify a novel series of molecules possessing similar modes of action, but improved pharmaceutical properties compared with ON01910.Na, we have designed and synthesized a class of (E)-styrylsulfonyl methylpyridines. One of the lead compounds with improved oral bioavailability (F = 56% oral dosing at 10 mg/kg in mice; Supplemental Table 1) (Lu et al., 2014), designated as TL-77 [(E)-N-(2-methoxy-5-((2,4,6-trimethoxy styrylsulfonyl)methyl)pyridin-3-yl) methanesulfonamide] (Fig. 1), has been investigated for its cellular mechanism of action. Utilizing biochemical and cell-based assays, we show that TL-77 arrests cells at the G2/M phase in cancer cells, followed by the onset of apoptosis. Treatment of HCT-116 cells with TL-77 inhibits mitotic spindle assembly, evoking formation of multipolar spindles and misalignment of chromosomes, which is similar to the phenotype observed with Plk1-depleted cells. In cell-free conditions, TL-77 potently inhibits tubulin polymerization. Mitotic markers, Cdc25C and cyclin B1, were investigated to explore the molecular consequences of TL-77 treatment. Phosphorylation of Cdc25C (Ser198) has been suggested to serve as a biomarker to track inhibition of Plk1. TL-77 inhibits phosphorylation of Cdc25C and downregulates cyclin B1 in cancer cells. These observations reveal that TL-77 induces spindle abnormalities and may suppress the activity of Plk1 in cancer cells. TL-77 causes downregulation of antiapoptotic proteins, and induces caspase-dependent apoptosis, which are consistent with the effects of ON01910.Na. Unlike ON01910.Na, however, TL-77 causes preferential toxicity in cancer cells when compared with normal cells. PI3K/Akt/mammalian target of rapamycin (mTOR) signal transduction is minimally disturbed in tumor cells after TL-77 treatment.

Materials and Methods

Inhibitor Compounds. TL-77 was synthesized in our laboratory as reported previously (Lu et al., 2014), and the purity and structure confirmation is reported in Supplemental Method 1. ON01910.Na was purchased from Allichem LLC (Baltimore, MD).

Cell Culture. All cell lines were obtained from the American Type Tissue Culture Collection, expanded, and stored in the liquid nitrogen cell bank at the Centre for Biomolecular Sciences, University of Nottingham (Nottingham, UK). All cancer cell lines were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS). Human dermal microvascular endothelial cells (HMEC-1) were maintained in Endothelial Cell Growth Medium 2 with 10% FBS. Human umbilical cord vein endothelial cells (HUVECs) were maintained in Endothelial Cell Growth Medium 2 with 10% FBS. All cell lines were incubated at 37°C in a humidified, 5% CO₂ atmosphere.

Selection of Cell Lines. HCT-116 colorectal and A2780 ovarian cell lines were chosen to represent two common and intractable cancers, among the most common malignancies worldwide. They are commonly used as model systems for studying cancer pathways and for developing new therapeutic approaches. HCT-116 cells are well characterized by an intact DNA damage checkpoint and normal p53 responses (Vogel et al., 2004). Upregulated expression of seven genes responsible for DNA damage signaling was observed in HCT-116 cells, including Cdc25C (Duldulao et al., 2012). In addition, HCT-116 cells are reported to exhibit high Plk1 expression and activity (Herz et al., 2012). Both HCT-116 and A2780 cell lines have been validated in the scientific literature to be mitotic spindle checkpoint proficient (Birk et al., 2012). A2780 cells possess confirmed biallelic inactivation of phosphatase and tensin homolog (PTEN; 9 base pair deletion in exon 5 and 37 base pair deletion in exon 8) (Wu et al., 2011), and therefore, deregulated PI3K signal transduction (Burger et al., 2011). In view of intracellular effects and potential targets of ON01910.Na reported to date, these two cell lines were used in this study for investigation of the mechanism(s) of action of TL-77.

MTT Cytotoxicity Assays. Standard MTT (thiazolyl blue; 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were performed as reported previously (Wang et al., 2004). In brief, cells were seeded (1500–3000 cells/well) in 96-well plates and allowed to attach overnight. Cells were incubated for 96 hours with inhibitors using a range of concentrations to generate dose-response curves. The concentration required to cause 50% growth inhibition (IC₅₀) was determined using nonlinear regression analysis.

Cell Cycle Analysis and Detection of Apoptosis. Cell-cycle effects and induction of apoptosis were examined in cancer cells as well as nontransformed cells. Sample preparation, staining, and analyses were performed following the protocol provided by BD (BD Biosciences, San Jose, CA). HCT-116 and A2780 cells were seeded at a density of 6 × 10⁵. Following overnight incubation then treatment with inhibitors at appropriate concentrations and for specific time points, cells were collected, pelleted, and washed twice with cold (4°C) phosphate-buffered saline (PBS). For cell-cycle analyses, cells were centrifuged at 1200 rpm for 5 minutes at 4°C and resuspended in 0.4 ml of fluorochrome solution containing 50 μg/ml propidium iodide (PI) in 0.1% sodium citrate plus 0.1% Triton X-100, protected from light and stored at 4°C overnight.

For determination of apoptotic populations, annexinV/PI staining was used. First, 1 × 10⁶ cells were centrifuged at 1200 rpm at 4°C. Supernatants were discarded and the cell pellet was resuspended in 100 μl of binding buffer and 5 μl of annexin V–fluorescein isothiocyanate (FITC). Samples were incubated at room temperature for 15 minutes in the dark, and 400 μl of binding buffer was added, together with 10 μl of PI (50 μg/ml in PBS). Samples were kept on ice, protected from light for a further 10 minutes, and analyzed using fluorescence-activated cell sorting after vortex within 1 hour.

Cell-cycle analyses and quantification of apoptosis were conducted on a Beckman Coulter EPICS-XL MCL flow cytometer (Beckman Coulter Inc, Indianapolis, IN) and data analyzed using EXPO32 software (Beckman Coulter Inc).

Mitotic Index Assay. Mitotic index was determined using the Cellomics Mitotic Index Kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturer’s instructions. Fluorescence was measured at 460 nm at room temperature using a Leica DMIRE2 fluorescent microscope (Leica Microsystems, Wetzlar, Germany) to calculate the mitotic index, which is the percentage of cell nuclei stained with the...
mitosis-specific antibody (anti-phospho-histone H3) versus total cell nuclei stained with Hoechst. Vinblastine (0.5 μM) was used as a positive control, and dimethylsulfoxide (DMSO; 0.2%) was used as a negative control. Assays were carried out using duplicate plates.

**Caspase-3/7 Assay.** Caspase activity was determined using the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega, Madison, WI), following the manufacturer’s instructions. Fluorescence was measured at 480 nm at room temperature using an EnVision multilabel plate reader (PerkinElmer, Waltham, MA).

**Western Blot Analysis.** Western blot analyses were performed in a standard manner (Mahmood and Yang, 2012). The separated proteins were electroblotted to membranes (Bio-Rad Laboratories, Hercules, CA), and these were probed with specific primary antibodies (1° Abs). Anti-Akt, anti–phospho-Akt (Ser473), anti-Bid, anti-Bax, anti–Bcl-xl, anti-Cdc25C, anti–phospho-Cdc25C (Ser198), anticyclin B1, anticyclin D1, anti-Mcl-1, anti-mTOR, anti–phospho-mTOR (Ser2448), anti–poly (ADP-ribose) polymerase (anti-PARP), anti-phospho–phosphoinositide-dependent protein kinase 1 (PKD1) (Ser241), anti–phospho-PRAS40 (proline-rich Akt substrate of 40 kDa) (Thr246), and anti-PTEN Abs were obtained from Cell Signaling Technology, Inc (Danvers, MA). Anti–γ-H2AX (H2A histone family, member X) Ab was from Millipore, Inc (Darmstadt, Germany). Both antiamoine and antirabbit IgG horseradish peroxidase–conjugated 2° Abs were obtained from Dako (Dako Denmark A/S, Cambridge, UK).

**Tubulin Polymerization Assay.** The Tubulin Polymerization HTS Assay Kit (97% pure tubulin, porcine; Cytoskeleton, Denver, CO) was used according to the manufacturer’s protocol. The kit-based assay is an adaptation of a method (Rodriguez-Jimenez et al., 2010) where polymerization is monitored by optical density enhancement as it occurs. In brief, test compounds and their corresponding vehicles were pipetted into a 96-well microtiter plate, then tubulin reaction mixture (30 μM tubulin, 10 μM GTP, 100 μM MgCl2, and 80 mM PIPES [pH 6.9], 1 mM MgCl2, 1 mM EGTA, 1.0 mM GTP, and 2 mM sodium salt of highly purified porcine brain tubulin, 1:50 dilution) was added to each well, then incubated at room temperature and 37°C for 1 hour by recording fluorescence absorbance at 340 nm. The tubulin reaction mixture was composed of 80 mM PIPES (pH 6.9), 1 mM MgCl2, 1 mM EGTA, 1.0 mM GTP, and 2 mg/ml of highly purified porcine brain tubulin heterodimer.

**Immunofluorescence Microscopy.** HCT-116 cells (5 × 10⁶ cells/well) plated on 12-well chamber slides were treated without or with compounds for 24 hours. After treatment, cells were fixed in 3.7% formaldehyde in PBS for 15 minutes, and permeabilized with 0.3% Triton X-100 in PBS for 1 hour with blocking of nonspecific binding sites using 1% bovine serum albumin. Fixed cells were then incubated with anti–β-tubulin Ab (1:50 dilution) at room temperature and then exposed to the 2° Ab (FITC-conjugated goat antiamoine IgG at 1:100 dilution), followed by DNA staining with Hoechst. Photomicrographs were obtained using a Leica TCS SP2 Confocal Spectral Microscope.

### Results

**TL-77 Is a Potent Antiproliferative Agent.** MTT assays were used to assess the antiproliferative activity of TL-77 against a panel of 10 human tumor cell lines and three untransformed cell lines after 96-hour exposure (Table 1). Similar sensitivity was observed with cells for different p53 and retinoblastoma protein status for both compounds. TL-77 displayed potent antiproliferative activity with GI₅₀ (the concentration of TL-77 required to inhibit growth by 50% determined by MTT assay) values ranging between 317 and 875 nM in tumor cell lines irrespective of tissue origin. The two compounds were also tested against nontransformed cells, HMEC-1, HUVEC, and MRC-5. TL-77 exhibited modest selectivity: as illustrated in Table 1, TL-77 demonstrated >-2-fold greater potency in cancer cell lines over normal cells. Optimally, 10-fold greater selectivity was observed (A2780 GI₅₀ = 317 nM; MRC-5 GI₅₀ = 3144 nM). Overall, ON01910.Na was a more potent suppressor of tumor cell proliferation against the same carcinoma cell lines (GI₅₀ = 7–70 nM); however, little or no selectivity between the cancer and nontransformed human cells was detected (GI₅₀ = 30–51 nM in nontransformed cells). Time-dependent antiproliferative effects were further observed for both compounds as exemplified in HCT-116 cells (Fig. 2). Following 24-hour exposure to TL-77 or ON01910.Na, GI₅₀ values of 4 and 0.06 μM, respectively, in HCT-116 cells and 2 and 0.25 μM, respectively, in A2780 cells were observed and adopted as agent concentrations used in subsequent assays.

**TL-77 Induces Cancer Cell Cycle Arrest at G2/M.** By inhibiting Plk1 activity in cancer cells, ON01910.Na induces mitotic arrest characterized by spindle abnormalities, leading to apoptotic death (Gumireddy et al., 2005). Therefore, the effects of the two compounds on tumor cell-cycle progression were assessed by flow cytometry in two well characterized cancer cell lines, HCT-116 and A2780 (Fig. 3, A and B). These results showed stark and time-dependent G2/M cell-cycle arrest. TL-77 Induces Cancer Cell Cycle Arrest at G2/M.

### Statistical Analysis

All experiments were performed in triplicate and repeated at least twice. Student’s t tests for paired samples were used to determine statistical significance. *P* ≤ 0.05 was considered to be statistically significant.

### Table 1

Antiproliferative activity of TL-77 against a panel of cell lines

<table>
<thead>
<tr>
<th>Human Cell Lines</th>
<th>96 h-MTT, GI₅₀ ± S.D.</th>
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<tr>
<td></td>
<td>µM</td>
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<tr>
<td>Colon carcinoma</td>
<td>HCT-116 (p53+, Rb+)</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>HCT-116 (p53- , Rb+)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>MDA-MB 231 (p53- , ER- )</td>
</tr>
<tr>
<td>Ovarian carcinoma</td>
<td>MDA-MB 435 (p53- , Rb+)</td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td>A2780</td>
</tr>
<tr>
<td>Pancreatic carcinoma</td>
<td>PANC-1</td>
</tr>
<tr>
<td>Microvascular endothelial</td>
<td>HMEC-1</td>
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<tr>
<td>Umbilical vein endothelial</td>
<td>HUVEC</td>
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<tr>
<td>Lung Fibroblast</td>
<td>MRC-5</td>
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ER, estrogen receptor; Rb, retinoblastoma protein.
block (enhanced numbers of cells with 4 N DNA content) after treatment of both HCT-116 and A2780 cells with both compounds at GI50 values. Particularly in HCT-116 cells, TL-77 caused significant G2/M accumulation (>50%) as early as 6 hours, whereas ON01910.Na caused the same effect (∼50% G2/M arrest) after a longer exposure period (≥12 hour). Cell-cycle arrest was induced more rapidly when concentrations increased to 2 × GI50 for both compounds (effects in

Fig. 2. TL-77 and ON01910.Na display time-dependent antiproliferative effects in human cancer cells. MTT growth inhibition assay time-course experiments were carried out against human HCT-116 colon cancer cells. The data given are mean values derived from at least three replicates (n = 3 per experiment) ± S.D.

Fig. 3. TL-77 and ON01910.Na induce G2/M arrest in HCT-116 and A2780 cells. HCT-116 (A) and A2780 (B) cells were treated with GI50 concentrations of TL-77 or ON01910.Na. Cells were fixed, stained with propidium iodide, and DNA content was analyzed by flow cytometry.
HCT-116 cells are shown in Supplemental Fig. 1. Treatment of cells from both cell lines >18 hours resulted in accumulation of events containing sub-G1 DNA content, which is an indication of cell death, suggesting that the induction of apoptosis might be a consequence of cell-cycle arrest.

To further characterize the cell cycle effect after TL-77 exposure, a mitotic index assay which indicates the ratio between the number of cells in mitosis and the total number of cells was performed (Fig. 4). Both HCT-116 and A2780 cells were treated with TL-77 or ON01910.Na for a period of 6 or 12 hours. As shown in Fig. 4, A and C, TL-77 increased mitotic index in a time- and dose-dependent manner in both cell lines. Following 6-hour exposure to TL-77 (0.5–2 × GI50), 15–20% of cells resided in mitosis compared with <5% in control samples. An increased mitotic index (25%) was further observed in HCT-116 cells exposed to 4 × GI50 TL-77 (Fig. 4A). After 12-hour treatment with TL-77, a dramatic dose-dependent increase in mitotic index was observed in HCT-116 cells (>35%; 1–4 × GI50 TL-77), whereas a gradual climb of mitotic population was observed in A2780 cells (>35%; 4 × GI50 TL-77). Meanwhile, ON01910.Na caused less dramatic mitotic arrest in both cell lines as shown in Fig. 4, B and D. At concentrations >0.06 μM, a gentle dose-dependent increase in mitotic index was observed at both 6 and 12 hours. In HCT-116 cells, only at 4 × GI50 value (0.24 μM ON01910.Na) was a time-dependent divergence observed (mitotic index 17% and 27% after 6 and 12 hours, respectively).

Plots of mitotic indices (detailing percentage of mitotic cells in a given population) compared with data from cell cycle analyses (detailing G2/M events) are provided in Fig. 5. By subtracting mitotic indices from G2/M values, the population of cells in the G2 phase could be obtained. Whereas TL-77 evoked a dramatic mitotic block in both cell lines after 6-to 12-hour exposure (Fig. 5, A and C), the G2 block induced by TL-77 was less significant. In HCT-116 cells, numbers of TL-77-treated (4–8 μM) cells in the G2 phase remained stable at 6 hours; ~20% increased G2 events were observed when TL-77 exposure extended to 12 hours at 2 × GI50 (8 μM) (Fig. 5A). In contrast, ON01910.Na-treated cells revealed less significant mitotic blocks but a notable G2 arrest (Fig. 5, B and D). At 6 hours, ON01910.Na-treated HCT-116 cells started to arrest in the G2 phase (~10%) at 2 × GI50, whereas a steady raise was observed at both GI50 (~20%) and 2 × GI50 (~29%) in A2780 cells. Furthermore, after 12-hour treatment, ON01910.Na sharply increased the numbers of cells in the G2 phase (~20–30%) at both concentrations in both cell lines. These results are consistent with cell-cycle observations (Fig. 3A) where more rapid cell cycle arrest was observed in cells exposed to TL-77.

**TL-77 Inhibits Tubulin Polymerization.** A biochemical assay was adopted to then examine whether either compound affected tubulin polymerization (Fig. 6). Two known compounds, paclitaxel and nocodazole, were used as positive and negative controls, respectively. Paclitaxel binds to tubulin to suppress dissociation of microtubules (Diaz et al., 1998), and promotes polymerization of purified tubulin. In contrast, nocodazole inhibits polymerization by binding to β-tubulin and preventing formation of one of the two interchain disulfide linkages between tubulins (Vasquez et al., 1997; Chu and Ng, 2004). We compared the two test compounds’ effects on microtubule polymerization at GI50 and 1.5 × GI50 for TL-77, and GI50 and 2 × GI50 for ON01910.Na. As shown in representative traces in Fig. 6A, TL-77 acted (in a similar manner to nocodazole) as a microtubule suppressor at 4 and 6 μM. Similar results were obtained with ON01910.Na at 0.06 and 0.12 μM (Fig. 6B). These data indicate both compounds potently suppress tubulin polymerization in a manner similar to nocodazole at tested concentrations.

**TL-77 Perturbs Spindle Assembly in Cancer Cells.** To examine whether TL-77 could induce abnormal mitotic spindles, we examined the spindle architecture using confocal microscopy. Immunofluorescence staining using anti–phospho-histone H3 and anti–β-tubulin antibodies were performed in HCT-116 and A2780 cells. After 12-hour DMSO treatment, interphase cells were observed with bipolar mitotic spindles. As shown in Fig. 7, both 4 and 8 μM TL-77 caused remarkable inhibition of microtubule assembly in treated cells. The phenotypes caused by TL-77 included abnormal mitotic spindle formation with bi-, tri-, or multipolar spindles; chromosome misalignment; and complete loss of coordination in mitotic spindle assembly. Meanwhile, abnormal mitotic spindle formation was also observed in cells after ON01910.Na treatment. However, instead of generating...
multipolar spindles, cells exposed to ON01910.Na exhibited fragmented microtubules, resulting in prevention of cell division. Paclitaxel and nocodazole were used in this study as positive and negative controls (Supplemental Fig. 2). Cells treated with these two compounds displayed stabilized or depolymerized microtubules, respectively.

**TL-77 Downregulates Phosphorylation of Cdc25C.** In eukaryotic cells, the initiation of mitosis is triggered by activation of the Cdc2/cyclin B1 complex, the “mitosis-promoting factor” (Yang et al., 1998). Cdc25C, a substrate of Plk1, regulates Cdc2/cyclin B1 activity and controls entry into mitosis (Toyoshima-Morimoto et al., 2002; Schmit and...
Ahmad, 2007). Previous work reported that ON01910.Na inhibits activation of the phosphatase Cdc25C as one of its primary intracellular effects (Gumireddy et al., 2005; Jimeno et al., 2009). With emphasis on events upstream of Cdc25C, recent work identified the effect of ON01910.Na on DNA damage response signal pathways throughout the cell cycle (Oussenko et al., 2011).

Therefore, we next examined the effects of TL-77 on Cdc25C and cyclin B1 in HCT-116 and A2780 using Western blot analysis. Treatment with TL-77 for 8 hours dramatically reduced phosphorylation of Cdc25C at Ser198 in both HCT-116 and A2780 cells (Fig. 8). Similarly, ON01910.Na treatment also caused reduced levels of phosphor-Cdc25C. Although we found that both compounds minimally affected expression of cyclin B1 after 8-hour treatment in both cell lines, suppression of cyclin B1 was detected in TL-77–treated cells (16 μM) as well as ON01910.Na-treated cells (0.24 μM) after 24-hour treatment.

Inhibition of PI3K/Akt/mTOR Signal Transduction Caused by TL-77. ON01910.Na has been reported to inhibit PI3K/Akt/mTOR signal transduction as the major target with downregulation of cyclin D1 translation and activation of apoptosis (Prasad et al., 2009). To determine whether TL-77 modulates PI3K/Akt/mTOR signaling, we used Western blot to examine expression of key effectors of the Akt/mTOR pathway. HCT-116 and A2780 cells were treated with TL-77 in parallel with ON01910.Na. We first examined the effect of TL-77 on expression of the major cell-cycle regulator cyclin D1. As shown in Fig. 9, after 8-hour treatment, expression of cyclin D1 was significantly inhibited in ON01910.Na-treated cells at 0.24 μM (4 × GI50) and TL-77–treated cells at 8 μM (2 × GI50) in HCT-116 cells, whereas only minor inhibition of cyclin D1 expression was observed for both compounds at 4 × GI50 in A2780 cells.

Akt, a family of serine/threonine kinases, is the primary receptor of PI3K-initiated signaling and controls essential cellular activities through phosphorylation of a number of downstream effectors (Chang et al., 2003; Shaw and Cantley, 2006). Through PDKs, PI3K activates Akt, which in turn phosphorylates (thereby activating) mTOR protein at Ser2448. Western blot analysis detecting phosphorylated Akt Ser473 revealed that TL-77 (≥4 μM) significantly inhibited Akt phosphorylation in HCT-116 cells, suggesting that TL-77 negatively regulates Akt in the same manner as ON01910.Na (Fig. 9). To determine whether TL-77 inhibits effectors that function upstream of Akt, we examined phosphorylation of PDK1, which is known to have a central function in Akt activation (Osaki et al., 2004). Western blot analysis revealed that phosphorylation of PDK1 was downregulated in a dose-dependent manner by both compounds in HCT-116 cells. Complete inhibition of PDK1 phosphorylation was observed after 8-hour exposure of cells to 4 × GI50 TL-77 (16 μM) and ON01910.Na (0.24 μM) (Fig. 9). However, we found the treatment with both compounds for 8 hours minimally affected the phosphorylated Akt and PDK1 in A2780 cells.

The impact of TL-77 on downstream effectors of Akt was further investigated. One known Akt substrate is a 40-kDa, proline-rich protein (PRAS40) that binds mTOR to transduce Akt signals to the mTOR complex. Phosphorylation of PRAS40 by Akt at Thr246 helps to reduce PRAS40 inhibition of mTORC1 (Sancak et al., 2007). Although we found that ON01910.Na reduced phosphor-PRAS40 at 0.24 μM, which is first reported here, only minor inhibition of PRAS40 phosphorylation was
observed in TL-77–treated HCT-116 cells. This observation was confirmed in A2780 cells. Subsequently, we found that ON01910.Na treatment significantly reduced phosphorylation of mTOR in both cell lines. Although treatment with TL-77 for 8 hours did not induce changes in the phosphorylation status of mTOR in HCT-116, a dramatic inhibition was shown at 8 μM in A2780 cells. In addition, we observed that neither compound affected expression of PTEN in HCT-116 cells, the phosphatase major tumor suppressor protein and inhibitory regulator of PI3K/Akt signaling.

**TL-77 Induces Cancer Cell Apoptosis.** Induction of apoptosis was initially analyzed by annexin V/PI double staining in both HCT-116 and A2780 cancer cells following treatment with TL-77 for 24 and 48 hours (Fig. 10, A and B). Both TL-77 and ON01910.Na induced apoptosis in dose- and time-dependent manners in both cell lines. TL-77 induced HCT-116 apoptosis at GI50 and 2 × GI50 values (4 and 8 μM, respectively) ≥24-hour exposure with maximal effect after 48-hour treatment, whereas ON01910.Na triggered apoptosis more slowly than TL-77 (minor effect at 24 hours, clear effect at 48 hours). A2780 cells appeared more sensitive to ON01910.Na and TL-77; both TL-77 and ON01910.Na at GI50 effectively induced apoptosis with >30% (24 hours) and >60% (48 hours) of cells going through early apoptosis. These time-dependent observations are consistent with cell-cycle effects.

Molecular mechanisms of TL-77–mediated apoptosis were further explored in both cell lines. Western blot assays in HCT-116 cells (Fig. 11A) revealed that TL-77 (24-hour treatment) induced dose-dependent PARP cleavage ≥4 μM. However, consistent with insensitivity to apoptosis observed in the annexin V/PI dual staining assay, PARP cleavage was not observed in cells exposed to ON01910.Na <0.24 μM (4 × GI50). Consistent with annexin V–positive A2780 populations detected by flow cytometry (Fig. 10B), ON01910.Na-treated
cells (0.25–1.0 μM) showed significant PARP cleavage, whereas treatment with TL-77 indicated only minimal cleavage of PARP <8 μM (4 × GI50) (Fig. 11A).

PARP cleavage is the result of activation of caspase 3; therefore, a caspase 3/7 activation assay was performed in HCT-116 cells and HMEC-1 untransformed cells after 24-hour treatment with either TL-77 or ON01910.Na (Fig. 10C). These data demonstrate that concentrations ≥2 μM (0.5 × GI50) TL-77 dose dependently enhanced caspase 3/7 activity compared with DMSO control, confirming an active apoptotic process during this period, but no such activity was detected in HMEC-1 cells ≤20 μM (4 × GI50). In contrast, ON01910.Na significantly activated caspase 3/7 at >0.06 μM (GI50) in normal cell lines as well as in normal cells at 0.12 μM (2 × GI50).

Caspases and Bcl family proteins comprise the executors of apoptosis. As apoptosis related to Bcl family members is affected by the balance between proapoptotic (Bax, Bid) and antiapoptotic (Bcl-xl, Mcl-1) molecules (Adams and Cory, 2007), we tested expression levels of Bcl-2 family members in HCT-116 cells after 24-hour treatment. As shown in Fig. 11B, expression levels of Bid were significantly reduced in TL-77-treated cells compared with controls, whereas ON01910.Na treatment resulted in only a minor effect. Total protein levels of Bax in HCT-116 cells following treatment with these compounds were then examined; we observed that the expression of Bax was essentially unchanged. Expression of Bcl-xl and Mcl-1, however, was significantly reduced in TL-77-treated cells compared with controls. Treatment with TL-77 at 16 μM for 24 hours resulted in the complete inhibition of Bcl-xl protein expression.

**TL-77 Induces Histone H2AX Phosphorylation.** Cell-cycle checkpoints protect normal cells from tumorigenesis by delaying progression of the cell cycle until DNA is repaired. H2AX is a variant of histone H2A required to maintain genomic stability (Celeste et al., 2003). Phosphorylation of Ser139 of the histone human variant H2AX, commonly

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**Fig. 9.** Effect of TL-77 and ON01910.Na on the Akt/mTOR signal transduction pathway. HCT-116 and A2780 cells were incubated with indicated concentrations of TL-77 and ON01910.Na for 8 hours. The cells were lysed and subjected to Western blot analysis using anti-p-AKT antibody, anti-p-mTOR antibody, and indicated antibodies. Equal loading was confirmed in each lane by stripping and reprobing the blots with either anti-AKT antibody or anti-mTOR antibody. β-Actin was used as an internal control for protein loading. The Western blot results were quantified and statistical analysis was performed (*P < 0.05). All of the aforementioned experiments were repeated three times, and a representative result is shown. p-AKT, phosphor-AKT; p-PDK1, phosphor-PDK1; p-PRAS40, phosphor-PRAS40; p-mTOR, phosphor-mTOR.

---

**Table 1.** Western blot analysis of Akt/mTOR signaling in HCT-116 and A2780 cells treated with TL-77 and ON01910.Na for 24 hours. The blots were quantified using Image J software and normalized to β-Actin levels. The data are presented as mean ± SEM. *P < 0.05 compared to DMSO control. ' indicates significant difference between TL-77 and ON01910.Na treatment. All experiments were performed in triplicate, and a representative result is shown.

<table>
<thead>
<tr>
<th></th>
<th>HCT-116</th>
<th>A2780</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TL-77</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0.06</td>
<td>0.25</td>
</tr>
<tr>
<td>ON01910.Na</td>
<td>0.12</td>
<td>0.50</td>
</tr>
<tr>
<td>8 μM</td>
<td>16</td>
<td>8</td>
</tr>
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</table>

**Notes:** TL-77 and ON01910.Na were added to the cells for 24 hours. The blots were quantified using Image J software and normalized to β-Actin levels. The data are presented as mean ± SEM. *P < 0.05 compared to DMSO control. ' indicates significant difference between TL-77 and ON01910.Na treatment. All experiments were performed in triplicate, and a representative result is shown.

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**Fig. 10.** Western blot analysis of caspase 3/7 activation in HCT-116 and A2780 cells treated with TL-77 and ON01910.Na for 24 hours. The blots were quantified using Image J software and normalized to β-Actin levels. The data are presented as mean ± SEM. *P < 0.05 compared to DMSO control. ' indicates significant difference between TL-77 and ON01910.Na treatment. All experiments were performed in triplicate, and a representative result is shown.

**Fig. 11.** Western blot analysis of Bcl family proteins in HCT-116 cells treated with TL-77 and ON01910.Na for 24 hours. The blots were quantified using Image J software and normalized to β-Actin levels. The data are presented as mean ± SEM. *P < 0.05 compared to DMSO control. ' indicates significant difference between TL-77 and ON01910.Na treatment. All experiments were performed in triplicate, and a representative result is shown.
referred to as γ-H2AX, is an early cellular response to the induction of DNA double-strand breaks. Detection of this phosphorylation event has emerged as a highly specific and sensitive biomarker for monitoring DNA damage (Mah et al., 2010). Cellular expression of γ-H2AX was detected following treatment of HCT-116 cells with TL-77 or ON01910.Na for 8 and 24 hours, indicative of DNA damage. Western blot analysis of HCT-116 cells after treatment with TL-77 and ON01910.Na for 8 hours showed significant dose-dependent elevation of γ-H2AX (Fig. 11C). Following treatment of cells with either compound for 24 hours, intense bands revealed high levels of γ-H2AX. These data reveal that early and prolonged phosphorylation of histone H2AX has occurred after treatment with TL-77 or ON01910.Na.

Discussion

The majority of anticancer chemotherapy drugs are delivered by intravenous or oral routes which impact directly on bioavailability. Improvements in bioavailability have been found to enhance efficacy and tolerability and decrease adverse effects (Dmochowski and Staskin, 2002). By modifying the structure of ON01910.Na, we designed and synthesized TL-77, a novel (E)-styrilsulfonyl methylpyridine. Pharmacokinetic studies in mice revealed that oral bioavailability of TL-77 was improved to 56%, compared with 9% for ON01910.Na (Lu et al., 2014). Based on these findings, we hypothesize that the structural modification may influence positively on efficacy and tolerability. Therefore, in this study, we have
investigated in detail the cellular mechanisms of action of TL-77 in comparison with ON01910.Na. TL-77 displays potent growth inhibitory activity in vitro (GI50, 1 μM against colon, breast, ovarian, cervical, and pancreatic carcinoma as well as melanoma cell lines). Importantly, TL-77 demonstrates significant selectivity in tumor cell lines over nontransformed HMEC-1, HUVEC, and MRC-5 cells.

Cell-cycle and mitotic index analyses reveal that TL-77 evokes rapid mitotic arrest (6 hours) followed by delayed G2 block (12 hours, 8 μM) in HCT-116 cells. In contrast, cells exposed to ON01910.Na accumulate in the G2 cell-cycle phase, failing to traverse mitosis. TL-77, however, mediates a rapid mitotic inhibitory effect as an initial event.

Mitotic spindle assembly is critical for segregation of chromosomes to two daughter cells during mitosis. Several mitosis-specific protein kinases are required to organize the bipolar spindle assembly and chromosome biorientation, including Cdc2, aurora B, and Plk1 (Peters et al., 2006). Plk1 has been identified as a key player for G2/M transition and mitotic progression in both normal and tumor cells (Schmidt et al., 2006). In late G2 phase and mitosis, Plk1 is essential for centrosome maturation, separation, and mitotic spindle assembly (Eckerdt et al., 2005). Downregulation of Plk1 activity in tumor cells results in mitotic arrest characterized by spindle abnormalities and apoptosis (Gumireddy et al., 2005). Mitotic entry is triggered by a steep increase in cyclin B/Cdc2 activity (Lindqvist et al., 2009). Plk1 promotes the activation of cyclin B/Cdc2 by activating the phosphatase Cdc25C on Ser198 (Roshak et al., 2000). Cdc25C has been suggested to serve as a reliable marker to monitor Plk1 activity in a cellular context (Schmidt et al., 2006).

ON01910.Na has been reported to inhibit Plk1 and decrease expression of Cdc25C (Gumireddy et al., 2005; Jimeno et al., 2009). However, inconsistent effects of ON01910.Na on...
cycin B1 expression have been described (Jimeno et al., 2009; Prasad et al., 2009; Fantl et al., 2009). From our data, effective inhibition of phospho-Cdc25C but minor effects on cyclin B1 were observed after 8-hour treatment of both compounds in both cell lines. This indicates TL-77 suppressed Cdc25C activity, and the cyclin B1/Cdc2 complex still remained active. In addition, TL-77 evoked a notable mitotic block from mitotic index analysis (6- to 12-hour exposure). We therefore extrapolate that cells eventually entered mitosis during early stages of treatment (≤12 hours). It is more likely that cells become blocked at the mitosis checkpoint with the outcome of multipolar spindles and misaligned chromosomes. This led to a further conjecture that the target of TL-77 might be located at the kinase-mediating mitotic checkpoint, such as Plk1 (Barr et al., 2004), which still requires further clarification. After 24-hour treatment, suppressed expression of cyclin B1 was observed in ON01910.Na-treated cells (0.24 μM) as well as TL-77-treated cells (16 μM). Moreover, mitotic index analyses have shown increasing G2 arrest in cells after treatment with both ON01910.Na and TL-77 (2 × G2M only ≥ 12 hours). These data confirm the hypothesis that by inhibiting Cdc25C activity, both compounds maintain cyclin B/Cdc2 in an inactive state after longer exposure to compounds (12–24 hours). Consequently, cells fail to traverse mitosis and become blocked in the G2/M phase of the cell cycle. These observations indicate a multifaceted mechanism culminating in mitotic inhibition in cancer cells for both compounds. Immunofluorescence staining of α-tubulin revealed that TL-77 promoted disassociation of microtubules and inhibited mitotic spindle assembly, leading to formation of multipolar spindles and misalignment of chromosomes in vitro. This outcome is similar to the phenotype observed in Plk1-depleted cells (Sumara et al., 2004), suggesting TL-77 suppresses activity of Plk1 in cancer cells. In cell-free conditions, both ON01910.Na (0.06 and 0.12 μM) and TL-77 (4 and 6 μM) inhibited microtubule formation, acting in a similar manner to nocodazole. During experimental preparation, slight precipitation of TL-77 at 8 μM (2 × G2M) in H2O solution was observed. Consequently, a concentration of 6 μM (1.5 × G2M) was adopted. Minor inhibition or no effects of ON01910.Na on tubulin polymerization at 1 and 5 μM, respectively, has previously been reported (Gumireddy et al., 2005; Oussenko et al., 2011). These apparent inconsistencies could be due to experimental differences, such as testing buffer and compound concentrations. But the reproducibility between data sets in this study leads us to conclude that inhibition of tubulin polymerization is an important molecular mechanism of action of these agents. Although the antimitotic activity of ON01910.Na was deemed a consequence of Plk1 inhibition, the initial kinase inhibitory effects of ON01910.Na on Plk1 could not be reproduced by other scientists (Schmidt and Bastians, 2007; Steegmaier et al., 2007) nor by ourselves (Supplemental Table 2). In vitro kinase assays indicated that ON01910.Na failed to significantly inhibit Plk1 activity (<30 μM) (Steegmaier et al., 2007). Furthermore, several reports have confirmed that ON01910.Na inhibits tubulin polymerization; cells with depolymerized microtubules were observed after treatment with higher concentrations (2.5 μM) of ON01910.Na (Peters et al., 2006; Schmidt and Bastians, 2007; Steegmaier et al., 2007). Based on our observations, TL-77 as well as ON01910.Na inhibit tubulin polymerization, inducing microtubule abnormalities, and may suppress the activity of Plk1.

The PI3K/Akt pathway is a well characterized cell survival signaling pathway that blocks apoptosis and promotes survival and growth in cancer cells. From our results, TL-77, as well as ON01910.Na, downregulates cyclin D1 and inhibits phosphorylation of Akt and PDK1. Whereas ON01910.Na downregulates phosphor-mTOR is cell line–specific following treatment with TL-77 (A2780 only). Also, only ON01910.Na suppresses PRAS40 significantly in both cell lines. Taken together, these results indicate that TL-77 inhibits Akt/mTOR signal transduction; however, compared with ON01910.Na, arrest of Akt/mTOR signaling by TL-77 may be less significant and cell line–specific.

Dose- and time-dependent apoptosis induced by both compounds, detected by annexin V binding, is closely associated with induction of caspase 3/7 activity and PARP cleavage. TL-77 induced rapid apoptosis in HCT-116 cells, whereas ON01910.Na only triggered apoptosis after a longer exposure period, an observation consistent with cell-cycle effects, indicating that cell-cycle arrest may be the apoptotic trigger. Analysis of Bcl-2 family proteins after treatment of cells with TL-77 and ON01910.Na revealed that neither compound altered expression levels of the proapoptotic molecule Bax. However, expression of Bid was significantly decreased in cells following TL-77 treatment. This may indicate that the precursor form of Bid translocates to mitochondria, where it induces cytochrome c release (Luo et al., 1998), suggesting the involvement of the mitochondrial pathway in TL-77–mediated apoptosis. Among the prosurvival molecules, expression of Bcl-xL and Mcl-1 was significantly inhibited by both compounds. Taken together, these data show that TL-77 and ON01910.Na selectively induce G2/M cell-cycle block with ensuing induction of apoptosis in tumor cells. Preliminary cellular and mechanistic investigations demonstrate that TL-77 evokes excellent antiproliferative activity against a wide range of human tumor cell lines. TL-77 causes profound G2/M cell-cycle arrest with inhibition of Cdc25C phosphorylation, and induces multipolar spindles and chromosome misalignment in cancer cells, which leads to caspase-dependent apoptosis, effects comparable to those elicited by ON01910.Na. Compared with ON01910.Na, TL-77 elicits lower toxicity in nontransformed cells, possesses superior oral bioavailability, and mediates rapid mitotic inhibitory effects; moreover, TL-77 inhibits the Akt/mTOR survival signal transduction pathway as a secondary effect. These observations indicate a multifaceted mechanism culminating in mitotic inhibition in cancer cells. Therefore, further evaluation of this promising antitumor agent is justified.

Acknowledgements
The authors thank Dr. Hilary Collins from the Centre for Biochemical Sciences, University of Nottingham, UK, who provided the Hoechst dye and training for immunofluorescent staining.

Authorship Contributions
Participated in research design: Bradshaw, Lu, Laughton, Wang.
Conducted experiments: Lu.
Performed data analysis: Lu, Bradshaw, Laughton, Wang.
Wrote or contributed to the writing of the manuscript: Lu, Bradshaw, Laughton, Wang.

References


In vitro anti-tumour mechanism of 

(E)-N-(2-methoxy-5-(((2,4,6-trimethoxystyryl)sulfonyl)methyl)pyridin-3-yl)methanesulfonamide

Tiangong Lu, Charles A Laughton, Shudong Wang, Tracey D Bradshaw

Supplementary data

Supplemental Method 1.

Purity and structure confirmation of TL-77.

(E)-N-(2-Methoxy-5-(((2,4,6-trimethoxystyryl)sulfonyl)methyl)pyridin-3-yl)methanesulfonamide.

m.p. 211-212 °C. ¹H NMR (500 MHz, DMSO-­d₆) δ 2.96 (s, 3H, SO₂CH₃), 3.85 (s, 3H), 3.86 (s, 6H), 3.90 (s, 3H) (total 12H, 4 × OCH₃), 4.47 (s, 2H, CH₂), 6.29 (s, 2H, benzene-H), 7.12 (d, 1H, J 15.5, SO₂CH=CH), 7.54 (d, 1H, J 15.5, SO₂CH=CH), 7.65 (d, 1H, J 2.5, pyridine-H), 7.91 (d, 1H, J 2.0, pyridine-H), 9.25 (s, 1H, NHSO₂). ¹³C NMR (100 MHz, acetone-­d₆) δ 38.8, 53.3, 55.1, 55.5, 57.4, 90.7, 103.2, 119.4, 121.7, 123.2, 130.2, 134.7, 143.7, 154.1, 161.5, 164.3 (three carbon signals overlapping or obscured). HRMS (ESI) m/z 473.1115 [M+H]⁺; calcd. for C₁₉H₂₅N₂O₈S₂ [M+H]⁺ 473.1047. Anal. RP-HPLC: purity > 97%.
Supplemental Table 1.

Pharmacokinetic (PK) analysis of TL-77 in mice

TL-77 was subjected to pharmacokinetic (PK) analysis in mice following a single intravenous (IV) dose of 2 mg/kg or an oral (PO) dose of 10 mg/kg. ON01910.Na was included as a comparator. Blood samples were collected following decapitation at 0.030, 0.080, 0.25, 0.50, 1.0, 2.0, 4.0, 6.0, and 8.0 h. Plasma concentrations were used to calculate the PK parameters. TL-77 was shown to have excellent oral bioavailability with F = 56%, good half-life ($t_{1/2} = 3.02$ h) and AUC (> 3000 ng.hr/ml following PO administration). In contrast, ON01910.Na exhibited low oral bioavailability with an F = 9%; the half-life was shorter and drug exposure in plasma was lower compared to TL-77.

Table 1 Pharmacokinetic (PK) analysis of TL-77 in mice

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<th>Parameter (Unit)</th>
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<td>Exposure</td>
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<tr>
<td>F</td>
<td>56%</td>
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<td>9%</td>
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Supplemental Table 2.

Kinase Inhibitory Activity of TL-77 and ON01910.Na

We assessed the inhibitory activity of TL-77 and ON01910 against Plk1 and Aurora kinases. The two compounds are subjected to Merck Millipore’s KinaseProfiler™ service. Recombined kinases of interest are incubated in optimised assay buffer with validated substracts, 10 mM MgAcetate and [γ-33P-ATP]. The reaction is initiated by the addition of the MgATP mix. After incubation for 40 minutes at room temperature, the reaction is stopped by the addition of 3% phosphoric acid solution. 10 μL of the reaction is then spotted onto a P30 filtermat and washed three times for 5 minutes in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting. ATP concentration is used within 15 μM of the Km value for each kinase (Aurora-A 15 μM, Aurora-B 10 μM, Plk1 70 μM).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity±SD (% Control)</th>
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<tbody>
<tr>
<td></td>
<td>Aurora-A(h)</td>
</tr>
<tr>
<td>Control (Plus Enzyme)</td>
<td>100</td>
</tr>
<tr>
<td>Control(Blank)</td>
<td>/</td>
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<tr>
<td>TL-77 2μM</td>
<td>93±4</td>
</tr>
<tr>
<td>TL-77 4μM</td>
<td>95±6</td>
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<tr>
<td>TL-77 8μM</td>
<td>96±7</td>
</tr>
<tr>
<td>TL-77 16μM</td>
<td>112±3</td>
</tr>
<tr>
<td>ON01910.Na 0.03μM</td>
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<td>ON01910.Na 0.24μM</td>
<td>105±5</td>
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</table>
Supplemental Figure 1.

**TL-77 and ON01910.Na induce G2/M arrest in HCT-116 cells.**

Cells were treated with 2xGI₅₀ concentrations of TL-77 or ON01910.Na ≤ 24 h. Cells were fixed, stained with propidium iodide, and DNA content was analysed by flow cytometry.
Supplemental Figure 2.

Immunofluorescence staining of α-tubulin exhibited induction of spindle abnormalities by paclitaxel and nocodazole in cancer cells.

HCT-116 and A2780 cells are treated with DMSO, paclitaxel (1μM), or nocodazole (1μM) for 12 h, fixed with 4% paraformaldehyde, and treated with anti-α-tubulin antibody (green, FITC conjugated) and Hoechst (blue). The cells were analysed by confocal laser microscopy.