Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications.

Supporting Information

Probing Cytochrome P450-mediated activation with a truncated azinomycin analogue

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Chemicals & reagents

All chemicals were obtained from Aldrich (Poole, Dorset) and Lancaster (Morecambe, Lancashire). All solvents were supplied by Fisher. Silica for column chromatography: particle size 35-70 μm and thin layer chromatography plates (on aluminium) were supplied by VWR. Analytical thin-layer chromatography (TLC) was performed on plates precoated with silica gel 60 F254 (Merck). Visualisation of the plates was carried out using UV light (254 nm). Melting points were determined with a Stuart scientific SMP3 melting point apparatus. ^{1}H and ^{13}C NMR spectra were measured on a Bruker Advance AM 400 (400 MHz) spectrometer. NMR spectra were processed using a Bruker XWIN NMR 3.5 program. Low resolution mass spectra (LRMS) were generated using a Micromass Quattro Ultima mass spectrometer. High resolution accurate mass measurements were obtained from EPSRC National Mass Spectrometry Service Centre, University of Wales, Swansea. HPLC analysis was performed on Agilent Technologies 1200 HPLC system with diode array detection, using C18 reversed phase columns (Agilent Eclipse XDB - analytical: 4.6 x 100 mm; preparative: 21.2 x 150 mm). The purity of all compounds was ≥95%.

Synthetic protocols

Benzyl 3,3-dimethyloxirane-2-carboxylate *m*-CPBA (70% pure as supplied, 8.2 g, eq to 35 mmol) was added to a stirred mixture of benzyl 3-methylbut-2-enoate[1] (6.8 g, 35 mmol) and magnesium sulphate (10 g) in methylene chloride (100 mL). After 36 hours, more *m*-CPBA (70% pure as supplied, 2.0 g, eq to 17 mmol) was added and the stirring was continued for a further 48 hours. The solid was filtered and the filtrate was washed with 40% aqueous sodium hydroxide solution (100 mL), water (100 mL) and then dried over magnesium sulphate. Evaporation of solvent followed by chromatography on silica gel (20% v/v diethyl ether in petroleum ether) afforded the title compound as colourless oil (6.7 g, 93%). The spectroscopic characterisation was in agreement with that previously published for this compound.[2]

3,3-dimethyloxirane-2-carboxamide This compound was prepared in two steps as follows: A solution of toluene sulfonic acid monohydrate (0.5 g, 2.9 mmol) in toluene (100 mL) was

refluxed under a Dean-Stark trap to remove water azeotropically. Benzyl 3,3-dimethyloxirane-2-carboxylate (**5**) (5.51 g, 26.7 mmol) was added and the reflux was continued for a further 2 hours. Evaporation of solvent followed by chromatography on silica gel (20% v/v diethyl ether in petroleum ether) afforded benzyl 2-hydroxy-3-methylbut-3-enoate as colourless oil (5.26 g, 95%). The spectroscopic characterization was in agreement with that previously published for this compound.[1]

Aqueous ammonia (0.88 sp gravity, 100 mL) was added to benzyl 2-hydroxy-3-methylbut-3-enoate (5.26 g, 25.5 mmol) and the mixture was stirred at room temperature for 24 hours. The clear solution was stripped of solvent and purified by chromatography on silica gel (60% v/v ethyl acetate in petroleum ether to remove benzyl alcohol and then neat ethyl acetate) to afford the tile compound as a white solid (1.98 g, 69%).

δ_H (400 MHz, $CDCl_3$) 1.76 (3H, s, CH_3), 3.55 (1H, s, OH), 4.56 (1H, s, CH), 5.09 (1H, s, CH), 5.17 (1H, s, CH), 6.00 (1H, bs, NH), 6.11 (1H, bs, NH); δ_C [1] (100 MHz, $CDCl_3$) 16.7 (CH_3), 75.9 (CH), 116.0 (CH_2), 143.9 (C), 174.2 (C); HRMS: Found 115.0663 calculated for $C_5H_9NO_2$ 115.0633; m/z (ES) 115 (M^+, 100); ν_{max}/cm^{-1} 3328 (OH), 2923, 1670 (C=O), 1375, 1062, 1032, 1018, 906;

Synthesis of 5a as a typical procedure for the preparation of target compounds (5): DCC (1.15 g, 5.58 mmol) was added to a stirred mixture of compound **6** (430 mg, 3.72 mmol), naphthoic acid **7**a (942 mg, 4.36 mmol) and dimethylaminopyridine (90 mg, 0.75 mmol) in anhydrous methylene chloride (100 mL). After 4 days, solid (dicyclohexyl urea) was filtered and the filtrate was concentrated under vacuum and chromatographed on silica (50% v/v ethyl acetate in petroleum ether) to afford compound **5a** as a white solid (110 mg, 94%).

δ_H (400 MHz, $CDCl_3$) 1.87 (3H, s, CH_3), 2.60, (3H, s, CH_3), 3.94 (3H, s, CH_3), 5.13 (1H, m, CH), 5.28 (1H, s CH), 5.79 (1H, s, CH), 5.91 (1H, bs, NH), 6.09 (1H, bs, NH), 7.27 (2H, m, aromatic CH), 7.40 (1H, d, J = 3 Hz, aromatic CH), 7.77 (1H, d, J = 3 Hz, aromatic CH), 8.52 (1H, t, J = 5Hz, aromatic CH); δ_C (100 MHz, $CDCl_3$) 18.6 (CH_3), 20.1 (CH_3), 55.6 (CH_3), 77.3 (CH), 108.2 (CH), 117.1 (CH_2), 121.9 (CH), 123.7 (CH), 125.2 (CH), 126.8 (C), 127.9 (CH), 128.5 (C), 133.3 (C), 134.4 (C), 139.2 (C), 155.82 (C), 165.4 (C), 170.4 (C). ν_{max}/cm^{-1} 3395 (NH), 3192 (NH), 2967, 2936, 1729 (C=O ester), 1666 (C=O amide), 1615, 1598, 1414, 1272, 1205, 1188, 1087, 1049, 902, 848, 809. HRMS Found 313.1315, $C_{18}H_{19}NO_4$ req. 313.1314.

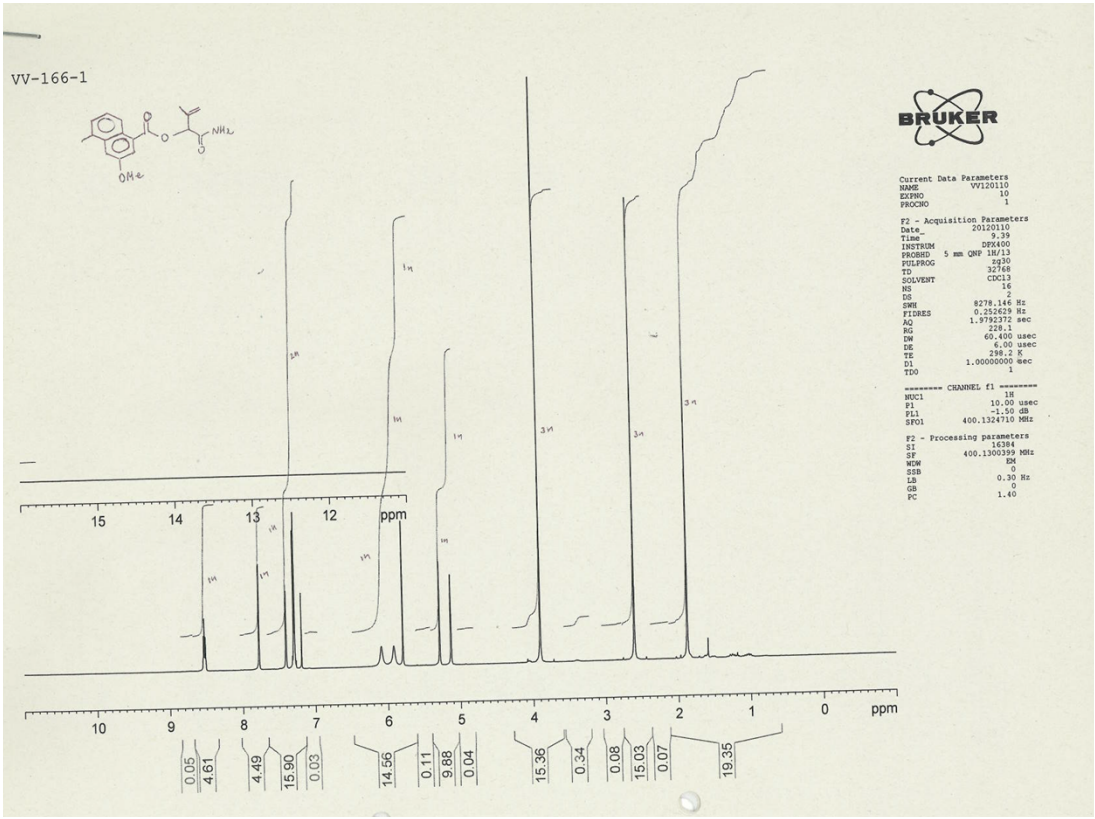


Figure S1: 1H-NMR of 5a

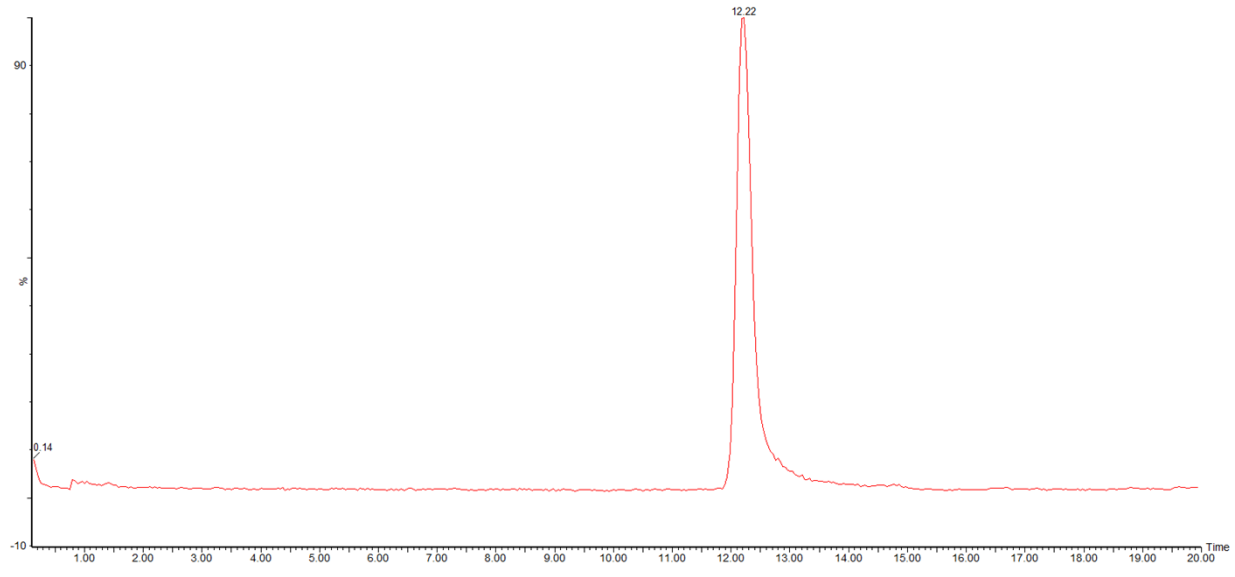


Figure S2: HPLC chromatogram of 5a

Compounds **5b** (96%), **5c** (92%), **5d** (78%) and **5e** (90%) were prepared similarly.

Typical procedure for the preparation of 3 and 4: *m*-CPBA (70% pure as supplied, 549 mg, eq to 2.3 mmol) was added to a stirred mixture of compound **5a** (700 mg, 2.3 mmol) and magnesium sulphate (100 mg) in methylene chloride (10 mL). After 15 h, more *m*-CPBA (550 mg) and magnesium sulphate (100 mg) were added. The solid was filtered and the filtrate was concentrated under vacuum and chromatographed on silica (40% v/v ethyl acetate in petroleum ether) to afford pure compound **3a** as a white solid (R_f = 0.45, 348 mg, 46%) and pure compound **4a** as a white solid (R_f = 0.40, 204 mg, 27%) as well a mixture of compounds **3a** and **4a** (154 mg).

The spectroscopic characterisation was in agreement with that previously published for this compound[3-4]: Compound **3a** δ_H (400 MHz, $CDCl_3$) 1.56 (3H, s, CH_3), 2.67 (3H, s, CH_3), 2.80 (1H, d, J= 4.5 Hz, H), 3.02 (1H, d, J= 4.5 Hz, H), 3.94 (3H, s, CH_3), 5.23 (1H, s, CH), 5.75 (1H, s NH), 6.11 (1H, s, CH), 7.27 (2H, m, aromatic CH), 7.40 (1H, d, J = 3 Hz, aromatic CH), 7.87 (1H, d, J = 3 Hz, aromatic CH), 8.55 (1H, t, J = 5Hz, aromatic CH). Compound **4a** δ_H (400 MHz, $CDCl_3$) 1.57 (3H, s, CH_3), 2.68, (3H, s, CH_3), 2.82 (1H, d, J= 4.5 Hz, H), 3.12 (1H, d, J= 4.5 Hz, H), 3.94 (3H, s, CH_3), 5.31 (1H, s CH), 6.06 (1H, bs, NH), 6.19 (1H, bs, NH), 7.27 (2H, m, aromatic CH), 7.40 (1H, d, J = 3 Hz, aromatic CH), 7.77 (1H, d, J = 3 Hz, aromatic CH), 8.57 (1H, t, J = 5Hz, aromatic CH).

Cell culture and chemosensitivity evaluation

CHO lines were a gift from the late Dr T Friedberg, University of Dundee and used as previously described.[5] The Flp-In™ system (Invitrogen) was used for the generation of the colon cancer SW480-2W1 cell line[6] and the gastric cell lines SGC-7901 and MKN-45 were obtained from the Type Culture Collection of Chinese Academy of Sciences. Compounds were dissolved in DMSO and then diluted in complete cell culture medium to give a broad range of concentrations (0.001-100 μM), such that the final DMSO concentration was not greater than 0.1%. Medium was removed from each well and replaced with compound or control solutions, and the well plates were then incubated for a further 96 h before the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. All three cell lines were grown as monolayers (1×10^4 cells) in DMEM at 37 °C in 5% CO_2. Results were expressed in terms of

IC_{50} values (concentration of compound required to kill 50% of cells) and all experiments were performed in triplicate.

Role of Cytochrome P450 (CYP) in chemosensitivity of 5a Involvement of specific CYP isoforms in the activation of **5a** was determined by evaluating the chemosensitivity of CYP-generated metabolites of **5a**. Metabolites were created via incubation of **5a** (50 μM) in the reaction mixture: (2 mM NADPH, 1 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4), 20 pmol of CYP1A1, 1A2, 1B1, 2D6 or 3A4 bactosomes (Cypex). Control reactions were carried out using CYP-null bactosomes. Following 1 h incubation at 37 °C, metabolites were extracted using acetonitrile and centrifugation at 10,000 g for 10 min. The resultant supernatant was removed, dried using vacuum evaporation (Genevac), and the resultant pellet resuspended in DMSO and the antiproliferative activity was assessed by the MTT assay following 96 h exposure to CHO cells as described above.

CYP1A1/1B1/3A4 bactosome metabolism of 5a using LC/MS

The presence of **5a** and the oxidised truncated azinomycin epoxides (**3a** and **4a**) were measured following incubation of **5a** for 180 min with CYP1A1, CYP1B1 or CYP3A4 bactosomes (6.3 pmoles P450/55 μg protein), NADPH (2 mM) and PBS (pH 7.4). At various time points between 0-180 min, a 20 μL sample was taken and the incubation was stopped by the addition of ice cold acetonitrile (40 μL) and centrifuged at 5000 rpm for 5 min. 10 μL of the supernatant was injected for analysis by LC/MS. LC/MS analysis was carried out using a Waters ZMD (Micromass, Manchester, UK) quadrupole mass spectrometer and Waters Alliance 2695 (Milford, MA, USA) quaternary pump chromatography system. The Waters Alliance 2695 is attached in series to the Waters ZMD mass spectrometer as described below. The mass spectrometer was operated in positive ion electrospray mode with a voltage of +3.00 kV applied to the capillary. A solvent flow of 1.0 ml/min (split 1:10) with a nitrogen gas flow of 400 l/min and a source temperature of 180 ºC was employed to produce stable spray conditions. The cone voltage was set at 25 V to give clear mass spectra from these samples. The mass spectra were continuously scanned from m/z 250 to m/z 700 per second throughout the entire HPLC separation. Masslynx software (V4.1, Micromass Ltd., Manchester, UK) was used to analyse the mass spectral data and produce total ion chromatograms (TICs) for the separation. Compound

5a (m/z 330) and hydroxylated metabolites **3a** and **4a** were monitored using UV/VIS (329nm) and a SIR (Single Ion Recording) channel.

Separation was achieved using gradient of 50% B to 70% B over 15 min rising to 90% between 15 and 25 min and remaining at 90% B until 30 min before returning to 50% B for re-equilibration (mobile Phase A contains 10% methanol 0.1% formic acid; mobile Phase B contains 90% methanol 0.1% formic acid). A flow rate of 1.0 ml/min was used throughout with a Hichrom RPB column (25 cm × 4.6 mm id) for the separation. A Waters 996 Photodiode Array Detector (γ = 330 nm) with Masslynx software (V4.1) was used for spectral analysis of the peaks of interest.

Quantitative real-time PCR (qRT-PCR)

Quantitative Real-Time PCR (qRT-PCR) was performed with SYBR® *Premix Ex Taq*™ Ⅱ (TaKaRa, China) with a StepOnePlus™ Real -Time PCR system (Applied Biosystems). The conditions for the PCR were 95°C for 30s, following by 40 cycles of 95°C for 5s and 60°C for 30s. The primers for all genes used in this experiment are listed in Table 1. The housekeeping *Gapdh* gene was used as indicator of the expression level of each gene, and relative gene expression data was calculated with the $2^{-\Delta\Delta Ct}$ method.

Table S1. Primer sequences, sizes of the PCR products and melting temperatures for Quantitative Real-Time PCR (qRT-PCR)

Gene	Primer	Sequence (5'to 3')	Product size (bp)	Melting temperature
Cyp1a1	Forward	CACCATCCCCCACAGCAC	75	60
	Reverse	ACAAAGACACAACGCCCCTT		
Cyp3a4	Forward	CACTCACCCTGATGTCCAGC	163	60
	Reverse	GACCCTCTCAAGTCTCATAGCAA		
Gapdh	Forward	CGGATTTGGTCGTATTGGG	208	60
	Reverse	CTGGAAGATGGTGATGGGATT		

Western Blotting Assay

Cells were harvested and suspended in RIPA Lysis Buffer (Beyotime).Protein concentrations were determined with Enhanced BCA protein assay kit (Beyotime), and bovine serum albumin was used as reference. 50μg of protein was separated on 10% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE), transferred to PVDF membranes, and probed with primary antibodies against CYP1A1 (Sangon biotech, AB20518b), CYP3A4 (Sangon biotech, AB20520a), GAPDH (Beyotime, AG019), β-Actin (Beyotime, AA128) respectively. Horseradish peroxidase-conjugated goat anti-mouse (Beyotime, A0216) and goat anti-rabbit (Beyotime, A0208) antibodies were used as the secondary antibody. Proteins were visualized with BeyoECL Plus (Beyotime).

Analysis of DNA damage - COMET assay

Sample Preparation

Cells were protected from direct light during the entire experiment to avoid light induced DNA damage to cells. Cells were seeded in 6-well plates at 5 x 105 cells per well in 2ml complete RPMI 1640 medium (supplemented with 10% fetal calf serum, 1mM sodium pyruvate and 2mM L-glutamine). HT29 cells were incubated under hypoxic conditions at 37ºC, 5% CO_2 and 0.1% O_2 and CHOWT cells were incubated under normoxic conditions at 37ºC, 5% CO_2 for 24 hours to allow cell attachment prior to drug exposure.

CHOWT cells were treated with either 50μM of **5a** (5A) or **5a**+CYP3A4 for 1 hour. Positive control compounds EO9, melphalan and camptothecin were also included for 1 hour exposure to cells at 20μM each. No recovery time was given to the cells after treatment.

Post treatment, cells were washed twice with HBSS and harvested by trypsinisation. For the detection of X-Links, cells were treated with 100μM of hydrogen peroxide (H_2O_2- DNA breakage agent) for 20 minutes immediately after treatment with test compounds. Cells were centrifuged at 2000g for 5 minutes and the pellet resuspended in 1ml of complete growth medium containing 10% DMSO. Samples were wrapped in several layers of paper towel and stored in -80ºC to enable gradual freezing until further processing.

Stock solutions were prepared at 10mM for EO9 and 1mM for camptothecin and melphalan in DMSO and stored at -20ºC. **5a** and **5a**+CYP3A4 were prepared in DMSO at 5mM and 1mM respectively and stored at -20ºC. Working solutions were diluted in complete medium.

Sample loading

The whole experiment was conducted in the dark. Cells were first resuspended in PBS, then embedded in low-melting point (LMP) agarose at 37ºC and this was transferred to normal melting point agarose-coated microscopic slides to which coverslips were added and the slides left on ice to polymerise. Once the agarose had solidified, coverslips were removed and another layer of LMP agarose was spread on the slides with coverslips and allowed to further polymerise.

Alkaline comet assay (SSBs and X-Links in DNA)

Coverslips were removed and slides were placed in ice cold lysis buffer (pH 10) for 1 hour at 4ºC. They were then submerged in electrophoresis buffer (pH>13) for 30 minutes to allow unwinding of the DNA and then subjected to electrophoresis (0.6V/cm) in the same electrophoresis buffer for 25 minutes. Neutralisation buffer was added to the slides 3 times for 5 minutes. Slides were then rinsed 3 times with dH_2O and then washed in 100% ice cold ethanol for fixation. Slides were left to air dry overnight.

Neutral comet assay (DSBs in DNA)

Coverslips were removed and slides were placed in lysis buffer (pH8) for 1 to 2 hours at 37ºC. Slides were then placed in electrophoresis buffer (pH8) 3 times at room temperature for 30 minutes and then subjected to electrophoresis (0.6V/cm) in the same electrophoresis buffer for 25 minutes. Slides were rinsed 3 times in dH2O and fixed in 100% ice cold ethanol. Slides were left to air dry overnight.

Scoring tail moments

Slides were stained with SYBRTMGOLD and images were taken through fluorescence microscope using Comet assay III software. A total of 50 randomly selected cells per sample were analysed.

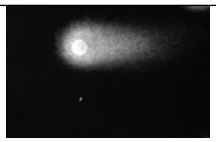
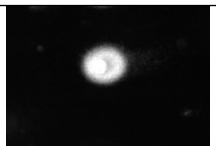
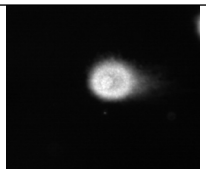
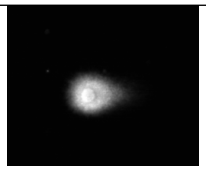
Type of DNA damage	Mean tail moment ± SD	Associated Images
DSB		
Positive control 20μM camptothecin	23.72 ± 7.92	
Negative control	9.62 ± 5.63	
50μM **5a**	5.93 ± 5.55	
50μM **5a** + 3A4	11.95 ± 3.90	

Figure S3: DNA double strand breaks – comet assay; for experimental protocol, see section "Analysis of DNA damage - COMET assay" in this supporting document.

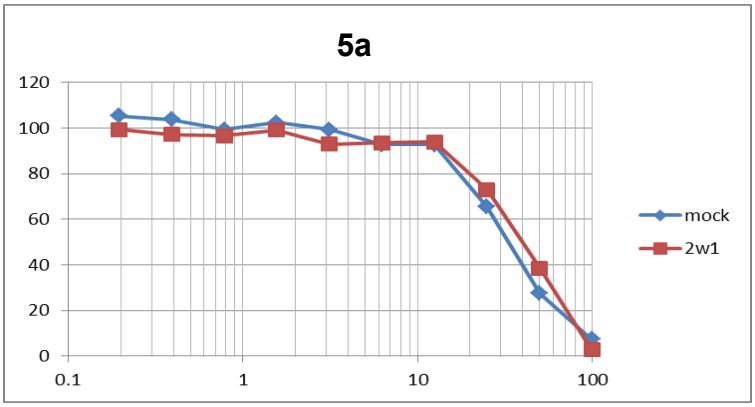


Figure S4: Antiproliferative activity of 5a (ICT2541) in mock and CYP2W1-transfected SW480 colon cancer cell lines; for experimental protocol, see section "cell culture and chemosensitivity evaluation" in this supporting document.

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(3) Alcaro, S.; Ortuso, F.; Coleman, R. S., Molecular modeling of DNA cross-linking analogues based on the azinomycin scaffold. *Journal of chemical information and modeling* **2005,** *45* (3), 602-9.
(4) Coleman, R. S.; Woodward, R. L.; Hayes, A. M.; Crane, E. A.; Artese, A.; Ortuso, F.; Alcaro, S., Dependence of DNA sequence selectivity and cell cytotoxicity on azinomycin A and B epoxyamide stereochemistry. *Org Lett* **2007,** *9* (10), 1891-4.
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(6) Travica, S.; Pors, K.; Loadman, P. M.; Shnyder, S. D.; Johansson, I.; Alandas, M. N.; Sheldrake, H. M.; Mkrtchian, S.; Patterson, L. H.; Ingelman-Sundberg, M., Colon cancer-specific cytochrome P450 2W1 converts duocarmycin analogues into potent tumor cytotoxins. *Clin Cancer Res* **2013**.