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Graphical abstract

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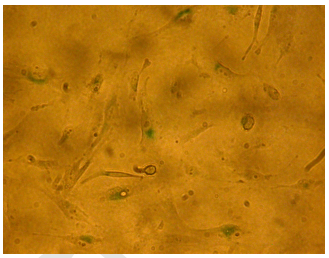

Legend: senescent cells after one week treatment with a combination of cis-platin and the acridine compound AS1410

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G-quadruplex compounds and cisplatin act synergistically to inhibit cancer cell growth in vitro and in vivo

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Abstract

The ability of two structurally diverse telomeric G-quadruplex binding compounds to synergise the action of cis-platin has been investigated in two cancer cell lines. One compound is a trisubstututed acridine compound AS1410, a close analogue of BRACO-19, and the other is a non-polycyclic compound synthesised using click chemistry and containing two triazole rings. Both compounds produce growth arrest at sub-cytotoxic concentrations in the two cell lines (MCF7 and A549), with behaviour consistent with telomere targeting mechanisms. Synergistic behaviour was observed in both cell lines with both compounds in combination with cis-platin, but only when the ratio of AS1410:cis-platin is >1. In vivo tumour xenograft studies with the A549 lung cancer model and the trisubstituted acridine compound AS1410 showed only a modest antitumour effect when administered alone, but produced rapid and highly significant decreases in tumour volume when administered in combination with cis-platin.

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1. Introduction

The application of two or more drugs in combination can enhance anti-tumour effects and minimise toxicity to normal cells and the development or selection of resistant cells [1, 2]. Utilising multiple drugs in concurrent and sequential combination can also provide the means to destroy residual cancer cells, with the hope of preventing metastatic disease and eventual tumour re-growth. Combination chemotherapy is currently the most successful approach in the clinical management of cancer through the use of combinations of existing cytotoxic drugs such as cis-platinum, taxol, vincristine often together with newer molecular targeted agents. The findings that most advanced human cancers involve multiple genetic abnormalities [3], suggests that this strategy is of importance for continuing clinical advance [4]. We report here on studies with a combination of the well-established cytotoxic agent cis-platin, with two telomeric G-quadruplex-binding compounds. This is an approach that targets one of the major differences between normal and cancer cells, the elevated expression of the telomerase enzyme complex and consequent maintenance of telomere length in the majority of human cancers.

Telomerase is selectively activated in ca 80-85% of tumour cells and primary tumours [5, 6], and plays a major role in cellular immortalisation and tumorigenesis [7]. Their potential as anticancer targets have been demonstrated in vitro and in vivo [8, 9], and a synthetic oligonucleotide targeting the telomerase RNA template [10] is currently in clinical trial in humans. Clinical trials of telomerase therapeutic vaccines also suggest that telomerase is a viable target in human cancer [11]. Conventional telomerase inhibition by small molecules results in telomere shortening consequent to telomerase inhibition, followed by eventual senescence and apoptosis [8]. Telomerase also directly caps and stabilises telomere ends; uncapping and exposure of these ends is followed by activation of the DNA damage-response system and rapid senescence/apoptosis [12].

Telomerase action requires the substrate, telomeric DNA, to be single-stranded in order to be recognized by the telomerase RNA template prior to the catalytic step in the telomere elongation cycle. Inhibition of telomerase can be achieved in an indirect manner by inducing the telomeric DNA substrate to fold into four-stranded guanine quadruplex structures, using small molecule quadruplex-binding compounds [13]. The resulting folded substrate cannot hybridize with the telomeric RNA template and the ability of telomerase to catalyze the synthesis of further telomeric DNA repeats is inhibited. The majority of these compounds contain an extended planar aromatic chromophore, in common with conventional duplex

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DNA-intercalating drugs [14]. We have previously reported that several quadruplex stabilising compounds based on a trisubstituted acridine motif can produce rapid anti-tumour effects in cells and in xenografts [15-17] through several parallel mechanisms involving telomere uncapping [18-20], direct or indirect telomerase inhibition and shortening of telomere length, with the characteristic induction of senescence and of apoptosis. The G-quadruplex approach thus selectively targets both telomerase and telomere maintenance in cancer cells [21-23].

The present study extends this approach by examining whether stabilising quadruplex structures at the telomeres following exposure to sub-cytotoxic concentrations of quadruplex compounds could sensitize cancer cells to other existing anticancer agents. We have focussed on the classic multi-cancer cytotoxic agent cis-platin [24] since it also acts at guanine-rich sites on DNA [25], albeit by very different mechanisms. It has been previously shown that cis-platin binds to human telomeric quadruplexes, and stabilizes their structure [26, 27]. Thus we hypothesized that cis-platin and a G-quadruplex compound would each augment the action of the other at the DNA level, and that this could result in synergistic effects in cancer cells. Previous studies have found evidence for synergy at the cellular level between several cytotoxic agents and the G-quadruplex compounds BRACO-19 and RHPS4 [15, 16, 28], though not for cis-platin, and we have previously shown that taxol and BRACO-19 act synergistically in a human tumour xenograft model [15]. We report here on a series of studies with two structurally very dissimilar types of G-quadruplex compound (Figure 1) in order to examine whether these effects are inherent in the G-quadruplex approach to targeting telomeres, and find significant synergy with cis-platin both in cell culture and in vivo. We conclude from the data on them that the cellular and in vivo effects are not restricted to one particular class of G-quadruplex compound. One compound, CL-1, has been generated by click chemistry (29) and the other compound AS1410 (30) is a close analogue of BRACO-19. AS1410 had been previously selected as a potential lead compound for possible clinical development.

2. Materials and Methods

2.1 *Cell lines*

The human cancer cell lines, breast (MCF7), lung (A549) and normal human fibroblast line (WI38) were purchased from American Type Cell Culture (ATCC). Both MCF7 and A549 cells were maintained in Dulbecco's Modified Eagles Media containing 10% foetal bovine serum (Invitrogen, UK), 0.5 mg/ml hydrocortisone (Acros Chemicals, Loughborough,

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UK), 2 mM L-glutamine (Invitrogen, Netherlands), and non-essential amino acids 1X (Invitrogen, Netherlands), and incubated at 37°C, 5% CO_2. The WI38 cell line was maintained in Minimal Essential Medium. MCF7 and A549 cell lines were routinely passaged at 1:6 and WI38 at 1:3 ratios.

2.2 *Preparation of compounds*

The two compounds AS1410 and CL-1 were all analytically pure [29, 30] and were initially dissolved in 100% DMSO to make up 10 mM stock solutions. Stock solutions were diluted immediately prior to use to a 1 mM working concentration in filtered sterilized deionised water and 1% HCl. Cis-platin was purchased from Aldrich and used without further purification.

2.3 *Sulphorhodamine B (SRB) short-term cytotoxicity assay*

Short-term growth inhibition was measured using the SRB assay as described previously [15, 31]. Briefly, cells were seeded (4000 cells/wells) into the wells of 96-well plates in DMEM and incubated overnight as before to allow the cells to attach. Subsequently cells were exposed to freshly-made solutions of compoundand cis-platin [CFG1]at increasing concentrations and incubated for a further 96 hr. Following this the cells were fixed with ice-cold trichloacetic acid (TCA) (10%, w/v) for 30 min and stained with 0.4% SRB dissolved in 1% acetic acid for 15 min. All incubations were carried out at room temperature. The IC_{50} value, the concentration required to inhibit cell growth by 50%, was determined from the mean absorbance at 540 nm for each compound concentration expressed as a percentage of the control untreated well absorbance.

2.4 *Short-term combination studies*

Combination studies were initially performed using the SRB 96 hr assay with varying ratios of cis-platin and compound, to elucidate the optimum ratios of the two compounds. Plates were stained and data was obtained as before. Data from combination studies were analysed using the Calcusyn software (Biosoft, UK) to derive Combination Index (CI) values [32].

2.5 *Long-term combination growth inhibition studies*

1 x 10^5 cells were seeded in 75 cm^2 tissue culture flasks and exposed to appropriate concentrations of compounds as single agents and in combination. The concentrations were chosen according to individual CI values and IC_{50} values as determined in the CI analysis and SRB assay. Cells were grown in a final volume of 10 ml DMEM and incubated as described previously. Cells were exposed to compounds twice a week by replacing with fresh media

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containing drug on day 3. On day 7 media was removed and cells were washed with PBS once and trypsinised using 3 ml of trypsin. Cells were then pelleted and resuspended in 10 ml of DMEM and viability was determined with a haemocytometer. From this 1 x 10^5 cells were reseeded and the experiment was continued as described before for a total of four weeks.

2.6 *Staining for senescence-associated β-galactosidase activity*

Staining for β-galactosidase activity was carried out per the instructions of the supplier (Cell Signaling Technology, Inc., Beverly, MA). In brief, cells from long-term exposure studies were retrieved at the end of each week and seeded in 35 mm 6-well plates (Nunc A/S) at a density of 1 x 10^5 cells in 2 ml media and incubated overnight under standard conditions together with the appropriate concentration of the compounds under investigation. After an approximately 24 h incubation period, the growth medium was removed, and the cells were washed, fixed, and stained using the supplied staining solution [400 mM citric acid/sodium phosphate (pH 6.0), 1.5 M NaCl, 20 mM $MgCl_2$, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mg of X-gal (5-bromo-4-chloro-3-indolyl-â-D-galactopyranoside)], followed by incubation overnight at 37°C (5% CO_2). Cells were examined by light microscope (mag. 200-800X) and counted the next day for the characteristic senescence-associated development of blue coloration.

2.7 *Detection of cellular telomerase activity by the TRAP (telomere repeat amplification protocol) assay*

Telomerase activity was measured with an adaptation of the previously-described method [33]. Briefly treated MCF7 or A549 cells (both treated with drug and control untreated) were lysed and total protein was quantified using the Bradford assay. The TRAP assay was carried out in two steps with an initial primer-elongation step and subsequent PCR amplification of the telomerase products to enable detection. In part 1, a master reaction mix was prepared containing the TS forward primer (0.1 µg; 5'-AATCCGTCGAGCAGAGTT-3'; Invitrogen, Paisley, UK), TRAP buffer (20 mM Tris-HCl [pH 8.3], 68 mM KCl, 1.5 mM MgCl2, 1 mM EGTA, 0.05% v/v Tween-20), BSA (0.05 µg), and dNTPs (125 µM each). Protein (1 µg) was then incubated with this reaction mixture for 10 min at 30ºC. Following heat inactivation of telomerase at 92ºC for 4 min. and cooling to 0ºC, 10 µL of a PCR reaction mix containing ACX primer (0.1 µg; 5'-GTG[CCCTTA]3CCCTAA-3'; Invitrogen, Paisley, UK) and 2U Taq polymerase (RedHot, ABgene, Surrey, UK) was added to each tube to start the PCR protocol for part 2, with thermal cycling being carried out in 3 parts following an initial 2min. denaturing period at 92 C (33 cycles of 92ºC for 30 s, 55ºC for 30 s, 72ºC for 45 s). PCR-amplified reaction products were then run out on a 10% v/v PAGE gel and visualised by

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staining with SYBR Green I (Sigma). EC_{50} values were subsequently calculated by quantitating the TRAP product using a gel scanner and GeneTools software (Syngene, Cambridge, UK).

2.8 *In vivo tumour xenograft studies*

A549 tumour cells (ATCC, Manassas, VA) were harvested and implanted (5×10^6 cells in phosphate-buffered saline) in the right flank of nude athymic mice. Female *nu/nu* MF1 mice (5 to 6 weeks of age) were obtained from the Biological Research Facility, St George's Hospital, London. After engraftment of tumours, mice were randomised to treatment groups when mean tumour volume reached approximately 0.1 cm^3 – absolute tumour volume was calculated as $v = \pi/6000 \times (L \times H \times W)$; orthogonal tumour dimensions were captured twice weekly.

Groups of mice (n=8) received AS1410 alone (1 mg/kg bodyweight), cis-platin alone (6 mg/kg) or the combination of AS1410 (0.3 or 1 mg/kg with 6 mg/kg cis-platin). Mice received cis-platin twice (Day 0 and Day 7) or AS1410 once daily for nine days (Days 0-4 and then Days 7-10). A further group of mice (n=8) remained untreated and served as contemporaneous controls. All treatments were given by slow bolus intravenous injection via a lateral caudal vein. Following the last treatment, mice remained on study until relative tumour volume (V_n/V_0) had increased six-fold.

Relative tumour volume was defined as V_n/V_0 where V_n was the volume after *n* days of treatment and V_0 as the absolute tumour volume on the first day of treatment (Day 0) – relative tumour volume on Day 0 = 1. The time taken to reach a six-fold increase in relative tumour volume (V6T) was determined and T-C values were calculated; differences in T-C were analysed by the Mann-Whitney rank-sum test. The fractional volume method (32) was used to assess additive or synergistic effects for combination treatment.

All procedures were conducted in accordance with UKCCCR and Home Office Guidelines and the study design was subject to approval from the St George's Hospital Animal Ethics Review Panel.

3. Results

We report here on the cellular effects as single agents and in combination with cis-platin of a triazole-derived 'click chemistry' G-quadruplex stabilising compound CL-1 [29], (Figure 1)**,** and a derivative (AS1410) of the trisubstituted acridine compound BRACO-19 containing a more lipophilic side-chain [15-17, 30]. A small library of 'click chemistry' compounds had been previously found to selectively stabilize G-quadruplex structures, as

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shown by melting experiments with quadruplex and duplex DNA [29]. One of these compounds (CL-1) together with the acridine compound AS1410 was chosen for further study following initial screening in short-term cytotoxicity assays using human breast carcinoma (MCF7) and lung carcinoma (A549) cells. Table 1 shows short term cytotoxicity data for the panel of cells reported in this study. Selectivity towards cancer cells was confirmed by screening against a non-cancerous human fibroblast cell line (WI38). Compound CL-1 showed significant selectivity between cancer and somatic cells. Cis-platin on the other hand, showed no such selectivity. Combination studies using sub-cytotoxic levels of each compound were undertaken on CL-1 and AS1410 in the MCF7 and A549 cell lines, in each case with varying ratios of cis-platin to elucidate the optimum ratios of each pair. Data from these short-term (96 hr) combination studies were analysed to derive Combination Index (CI) values, based on the multiple drug-effect equation of the enzyme kinetic models originally derived by Chou and Talalay [32]. The equation, however, ascertains only the additive effect (CI=1) and cannot directly determine synergism or antagonism. Nevertheless, synergism (CI=<1) is considered to be a "more than expected" additive effect, and antagonism (CI=>1) as a "less than expected" additive effect. A classical isobologram, when the interaction between the two drugs are additive, was plotted and effect levels ED_{50}, ED_{75} and ED_{90} were derived (the effective dose for the concentration of drug to elicit a response in 50%, 75% and 90% of the cell population respectively). Cells were also analysed for the induction of senescence following combination treatment.

The results for compound CL-1 at ratios of 1:1, 1:2, 2:1, 1:3 and 3:1 (cis-platin:CL-1). CI values at doses eliciting response in 50%, 75% and 90% of the population are tabulated in Tables 2a, b for MCF7 and A549 cells. CL-1 was able to instigate a response that is more pronounced than a purely additive one, at constant ratios of 1:1, 1:2, 2:1 and 1:3 (cis-platin:CL-1). However when the relative amount of cis-platin was increased compared to CL-1 (3:1 of cis-platin:CL-1) there was an antagonistic effect. The results of these short-term combination experiments were used to guide the design of a series of long-term cell culture experiments with a larger range of cis-platin:drug ratios, and all having drug in at least 3-fold excess of cis-platin. Figure 2a shows that the optimum combination with CL-1 and cis-platin produced an effect on MCF7 population doublings that started to become apparent after just one week, whereas the effect of the same concentration of CL-1 alone only became apparent after two weeks. Figure 2b shows that the combination in the MCF7 line resulted in a large increase in the percentage of cells undergoing senescence compared to treatment with either CL-1 or cis-platin alone, with an estimated error of ± 1.5. Figure 2c shows that only the

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combination produces a significant decrease in telomerase activity in cells, one week after the onset of treatment.

The acridine compound AS1410 similarly produced rapid cell growth arrest in the MCF7 cell line (Figure 3a), at a 2 μM concentration (ie slightly below the IC_{50} value). Low concentrations of cis-platin < 0.2 μM, resulted in modest attenuation of the growth curves (Figure 3a). Combination of very low dose cis-platin and AS1410, the latter at a dose (0.7 μM) that by itself is too low to significantly affect growth, results in a rapid and almost complete halt to population doublings (Figure 3), a highly significant synergetic effect. An equivalent effect was evident in the A549 cell line (Figure 3b), again at concentrations (4 or 6 μM) where AS1410 alone does not have a significant effect on cell growth. The synergistic combination has only a modest effect on telomerase activity after one week (Figure 4a), whereas the combination results in a highly significant level of senescent cells being evident after one week (Figures 4b, c).

Combination treatment of AS1410 (1 mg/kg) and a sub-optimal dose of cis-platin (6 mg/kg) to mice bearing A549 xenografts, resulted in tumour regression and statistically significant growth delays when compared with either agent alone (Figure 5; Table 4). Administration of either agent alone or AS1410 at 0.3 mg/kg in combination with cis-platin gave a slight reduction in growth rate, but this did not achieve statistical significance. Fractional volume analysis (Table 5) of synergy or additivity of the combination of AS1410 (1 mg/kg) with cis-platin (6 mg/kg) showed that the combination response was synergistic (R>1.0). A single cure was observed in the combination treatment with AS1410 (1 mg/kg), although not following administration of the lower dose of 0.3 mg/kg.

4. Discussion

We have previously reported that triazole-derived quadruplex stabilising compounds have high selectivity for G-quadruplex DNA over duplex DNA and that they inhibit telomerase activity in an in vitro TRAP assay [29]. The present study shows that the structurally highly diverse compounds CL-1 and AS1410 can both produce long-term growth arrest when in combination with cis-platin at sub-cytotoxic concentrations for both components in two well-established cancer cell lines. This suggests that the effect is not a consequence of the particular structure of a compound, but is likely to be a more general one. The effect is associated with significant increases in senescence after just one week of dosage, whereas only modest decreases in telomerase inhibition were observed. Growth arrest occurs in a manner that has been previously observed with other telomerase inhibition studies, using

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active-site hTERT inhibitors [10], as well as with other G-quadruplex interactive agents [16-20]. However whereas with direct telomerase inhibitor approaches, growth arrest and senescence generally occur only after extended periods of time, with the present (and other) G-quadruplex agents, the effects are apparent after a much shorter time-period. The two compounds studied here show profound cell growth inhibition within two weeks (compound CL-1) and almost immediately after exposure (AS1410), in accord with the increases in the senescent cell population. Previous studies on several G-quadruplex compounds, including BRACO-19, have shown that they are effective competitors for the hPOT1 protein that binds to the single-stranded telomeric DNA overhang [17-19], and that this, together with more direct uncapping of a telomerase-telomere complex, elicits a rapid DNA damage response leading to a selective in vivo anticancer effect. It cannot be ruled out that the level of telomerase inhibition achieved after 1-2 weeks is sufficient to drive the sub-population of cells with short telomeres into senescence within this time-frame. The evidence from the present study suggests that the MCF7 cell line is more responsive to a synergistic approach than the A549 line, which may be a consequence of the longer mean telomere length (6.5kb) in this cell line [34] compared to telomeres in MCF7 cells (4 to 5kb) [28], although this remains to be more thoroughly explored with a wider range of compounds, and may not be the case in vivo.

The synergetic effects found here for both compounds in combination with cis-platin, are only when the ratio of compound:cis-platin is >1. However when the relative amount of cis-platin was increased (for example with 3:1 of cis-platin:CL-1) there was an antagonistic effect, which is significantly greater in A549 compared to MCF7 cells. This is in accord with a previous observation using the G-quadruplex agent RHPS4, with a large 4:1 excess of cis-platin [28].

Cis-platin exerts its anti-tumour properties through binding to DNA, forming specific adducts and preferentially reacts with the N7 position of guanine (G) or adenine (A) in DNA to form a variety of monofuntional or bifunctional adducts. Since human telomeric DNA comprises tandem repeats of G-rich DNA sequences $(TTAGGG)_n$, it has been suggested it is especially susceptible to interaction with cis-platin [26, 27, 35], and may explain why the action of quadruplex-binding compounds is potentiated by cisplatin. A low ratio of cis-platin to a quadruplex compound may allow G-rich telomeric quadruplex formation to preferentially occur, aided by compound binding, without the telomeric DNA residues being already bound to cis-platin. At higher concentration of cis-platin the telomeric guanines may be bound to cis-platin and thus sufficient may be unavailable to form a quadruplex arrangement, although the

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detailed mechanism of action at the molecular level remains to be elucidated. A previous study [28] showed that combining the quadruplex stabilising compound RHPS4 with cis-platin produces an antagonistic effect in MCF7 cells using a ratio of 1:4 (RHPS4: cis-platin); the present study suggests that by inverting the ratios of the two drugs this outcome can be effectively manipulated to result in synergy. Recent observations of synergy between RHPS4 and camptothecin [36] also suggest that sequence of administration is an important factor. The demonstration of synergy between two types of molecules (G-quadruplex ligands and cis-platin) that both target G-rich sequences suggests that to structurally diverse G-quadruplex ligands may together also show synergy. This possibility though, remains to be explored.

The highly significant (and rapid) synergistic effect observed in the in vivo study with the AS1410 and cis-platin combination is in agreement with the effects observed in cell culture, and suggest that G-quadruplex compounds are effective anti-tumour agents against solid tumours when combined with the classic cytotoxic agent cis-platin. The ability of G-quadruplex compounds to produce rapid decreases in tumour growth with no evident phenotypic growth delay is also in contrast to the classic model of telomerase inhibition, providing further support to the proposals that such molecules are in effect selectively targeting the telomeres of cancer cells and may therefore be useful agents against human cancers.

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Table 1 Short-term antiproliferative ability (IC_{50}), in µM for cis-platin, the triazole-derived compound CL-1 and the acridine compound AS1410 in A549, MCF7 and WI38 cell lines.

	Cis-platin	**CL-1**	**AS1410**
MCF7 (Breast)	0.7	3.6	2.3
A549 (Lung)	4.7	3.4	7.4
WI38 (Somatic)	1.0	11.9	>50

Table 2 Combination index values at different ratios of doses (with a range of concentrations) eliciting response in 50% (ED_{50}), 75% (ED_{75}), and 90% (ED_{90}) of (a) MCF-7 and (b) A549 cells treated with cisplatin and compound CL-1 for 96 hr exposures.

(a)

Combination Index	**Cis-platin +CL-1 (1:1)**	**Cis-platin +CL-1 (2:1)**	**Cis-platin +CL-1 (1:2)**	**Cis-platin +CL-1 (1:3)**	**Cis-platin +CL-1 (3:1)**
ED_{50}	0.51087	0.53305	0.45938	0.41218	1.92009
ED_{75}	0.49719	0.33818	0.28144	0.4854	2.08635
ED_{90}	0.4983	0.21805	0.18106	0.60824	2.29252

(b)

Combination Index	**Cis-platin +CL-1 (1:1)**	**Cis-platin +CL-1 (2:1)**	**Cis-platin +CL-1 (1:2)**	**Cis-platin +CL-1 (1:3)**	**Cis-platin +CL-1 (3:1)**
ED_{50}	0.41292	0.64163	0.66164	0.65369	0.90413
ED_{75}	0.22032	0.48312	0.43528	0.35645	0.83236
ED_{90}	0.12404	0.38759	0.30430	0.19549	0.76708

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Table 3. Relative A549 tumour volumes (Mean ± SD) for selected days following intravenous treatment of mice with AS1410 (1 mg/kg on Days 0-4 and 7-10), cis-platin (6 mg/kg on Day 0 and 7), or combinations of AS1410 (0.3 mg/kg or 1 mg/kg) and cis-platin (6 mg./kg)

Day	**Control**	**AS1410** (1 mg/kg)	**Cis-platin** (6 mg/kg)	**AS1410 and cis-platin** (0.3 and 6.0 mg/kg respectively)	**AS1410 and cis-platin** (1.0 and 6.0 mg/kg respectively)
3	1.61 ± 0.337	1.30 ± 0.453	1.23 ± 0.264	1.56 ± 0.494	1.18 ± 0.221
9	2.18 ± 0.630	2.21 ± 0.852	1.57 ± 0.329	1.89 ± 0.675	1.24 ± 0.044
17	3.58 ± 1.656	2.73 ± 1.188	2.08 ± 0.597	2.01 ± 0.816	0.95 ± 0.257
23	3.87 ± 1.334	3.13 ± 1.786	3.25 ± 1.724	2.91 ± 1.205	1.03 ± 0.784
29	4.71 ± 1.306	4.24 ± 1.881	3.41 ± 1.344	3.88 ± 1.862	1.39 ± 1.080
Median V6T (days)	35	37	54	50	59
T-C (days)		+2	+19	+15	+24
p-value	-	NS	NS	NS	0.036 ($p<0.05$)

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Table 4. Fractional tumour volume analysis of AS1410 (1 mg/kg on Days 0-4 and 7-10), cisplatin (6 mg/kg on Day 0 and 7), or combination of AS1410 (1 mg/kg) and cis-platin (6 mg./kg)

	Fractional tumour volume				
Day	**AS1410**	**Cis-platin**	**Combination**		**R**
			(expected)	(observed)	
3	0.810	0.764	0.619	0.732	0.845
9	1.011	0.718	0.727	0.567	1.281
17	0.762	0.580	0.442	0.265	1.667
23	0.810	0.841	0.681	0.266	2.557
29	0.901	0.724	0.653	0.296	2.205

FTV (fractional tumour volume) = mean relative tumour volume (test) / mean relative tumour volume (control)

Expected FTV (combination) = FTV (compound 9) * FTV (cisplatin)

Ratio (R) = Expected FTV (combination) / Observed FTV (combination)

R values > 1.0 indicates synergy; R values < 1.0 indicates less than additive effects

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Figure legends

Figure 1. Structures of compounds used in this study.

Figure 2(a) Plots of the time-course of cell growth for MCF7 cells with respect to population doublings, following long-term exposure to cis-platin, compound CL-1 and various combinations of the two. The vehicle control experiment is marked "vc". **(b)** Percentage of senescing MCF7 cells treated with CL-1 and cis-platin from the long term combination growth inhibitory study. **(c)** Telomerase inhibition measured in MCF7 cells using the TRAP procedure, shown as a percentage decrease in activity, for various combinations of compound CL-1 and cis-platin, following a 1-week exposure.

Figure 3(a) Plots of the time-course of cell growth with respect to population doublings for MCF7 cells, following long-term exposure to cis-platin, AS1410 and various combinations of the two compounds. **(b)** Plots of the time-course of cell growth with respect to population doublings for A549 cells, following long-term exposure to cis-platin, AS1410 and various combinations of the two compounds.

Figure 4(a) Telomerase activity in A549 cells following a one week treatment with AS1410, cis-platin or the combination of both drugs using the TRAP procedure, shown as a percentage decrease in activity. **(b)** Percentage of senescence in A549 cells following one week treatment with AS1410, cis-platin or the combination of both drugs. **(c)** A549 cells stained for β-galactosidase activity following one week treatment with AS1410, cis-platin or the combination of both drugs. Panel A shows untreated cells, and panel B and C shows cells treated with 6μM AS1410 + 1μM cis-platin at x100 and x200 magnification respectively.

Figure 5 Relative growth curves of tumour volume with respect to time for the A549 xenograft model, for exposure to cis-platin (at a dosage of 6 mg/kg), AS1410 (at a dosage of 1 mg/kg) and two different dosages of AS1410 (0.3 and 1 mg/kg) in combination with cis-platin at a dosage of 6 mg/kg. Estimated standard deviations are given in Table 3; they have been omitted from the growth curves for reasons of clarity.

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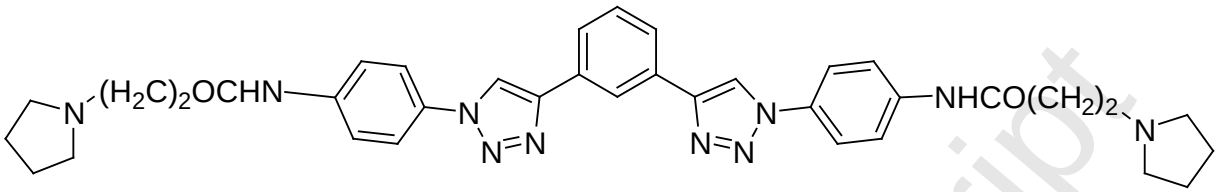
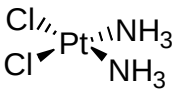
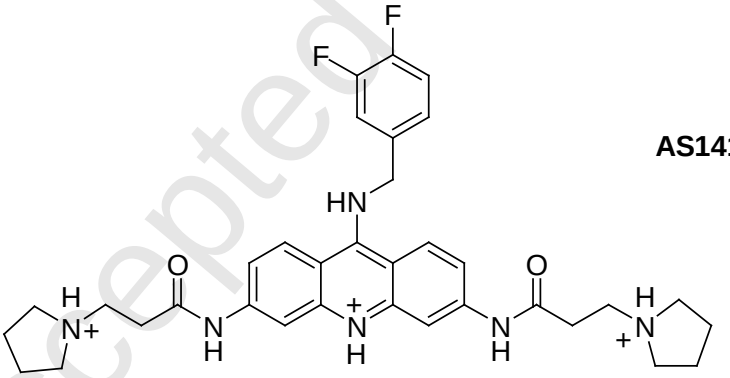
Figure 1



Click compound CL-1



cis-platin



AS1410

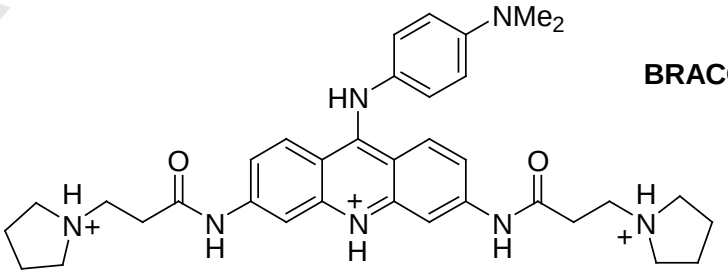


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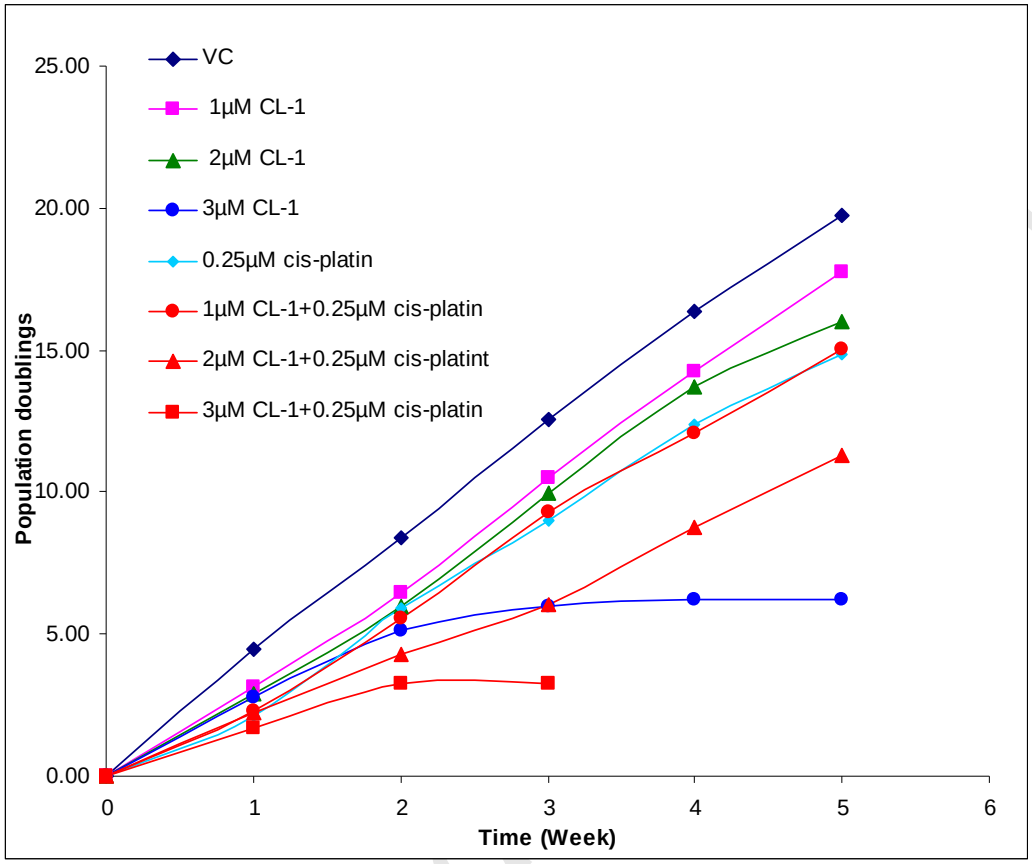
Figure 2a



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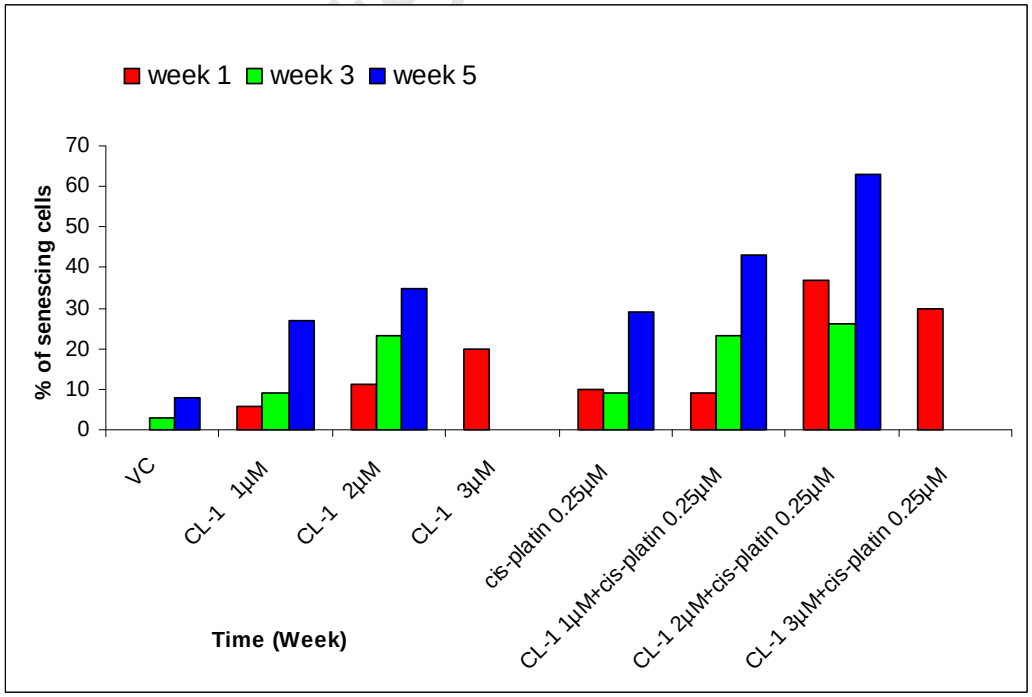

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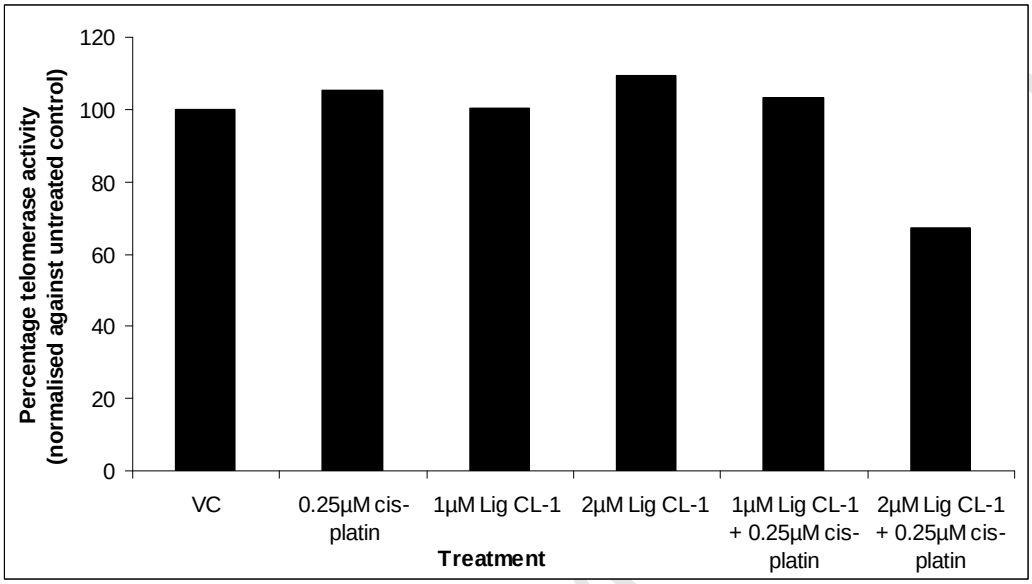

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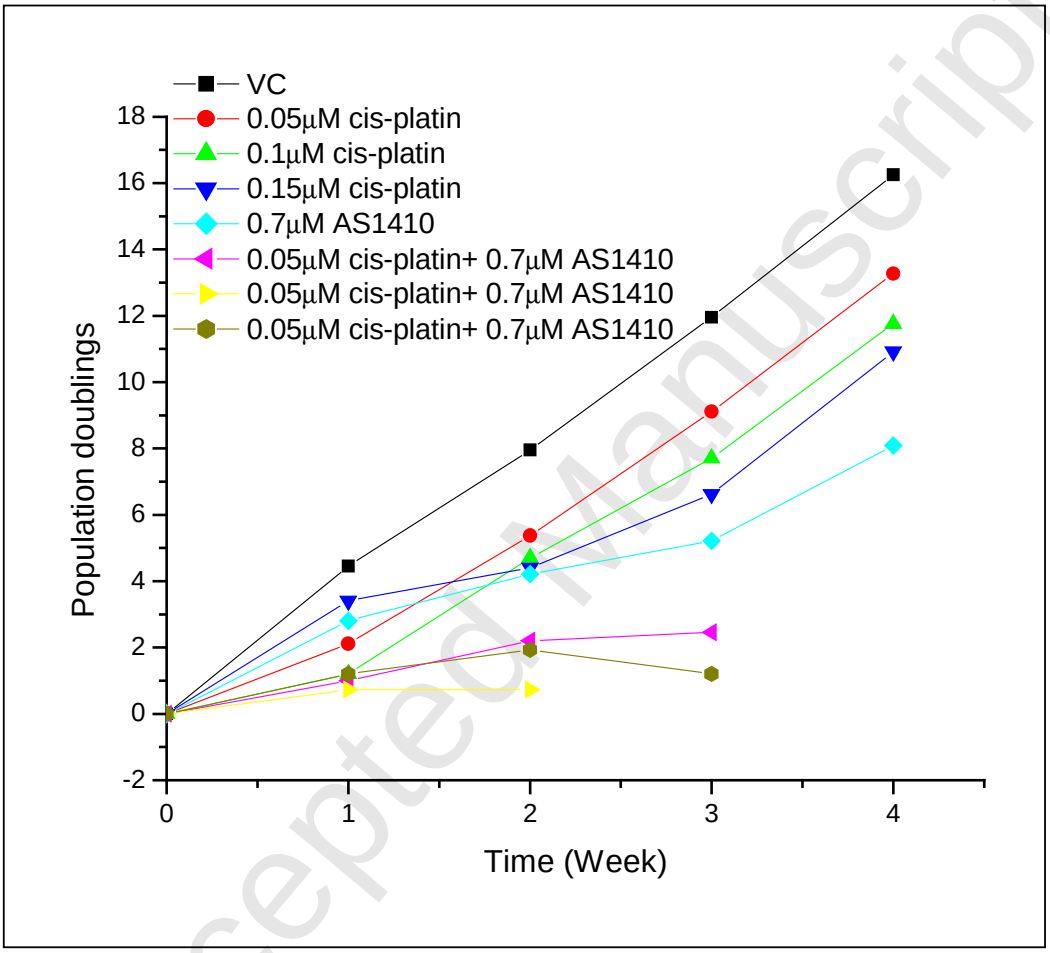

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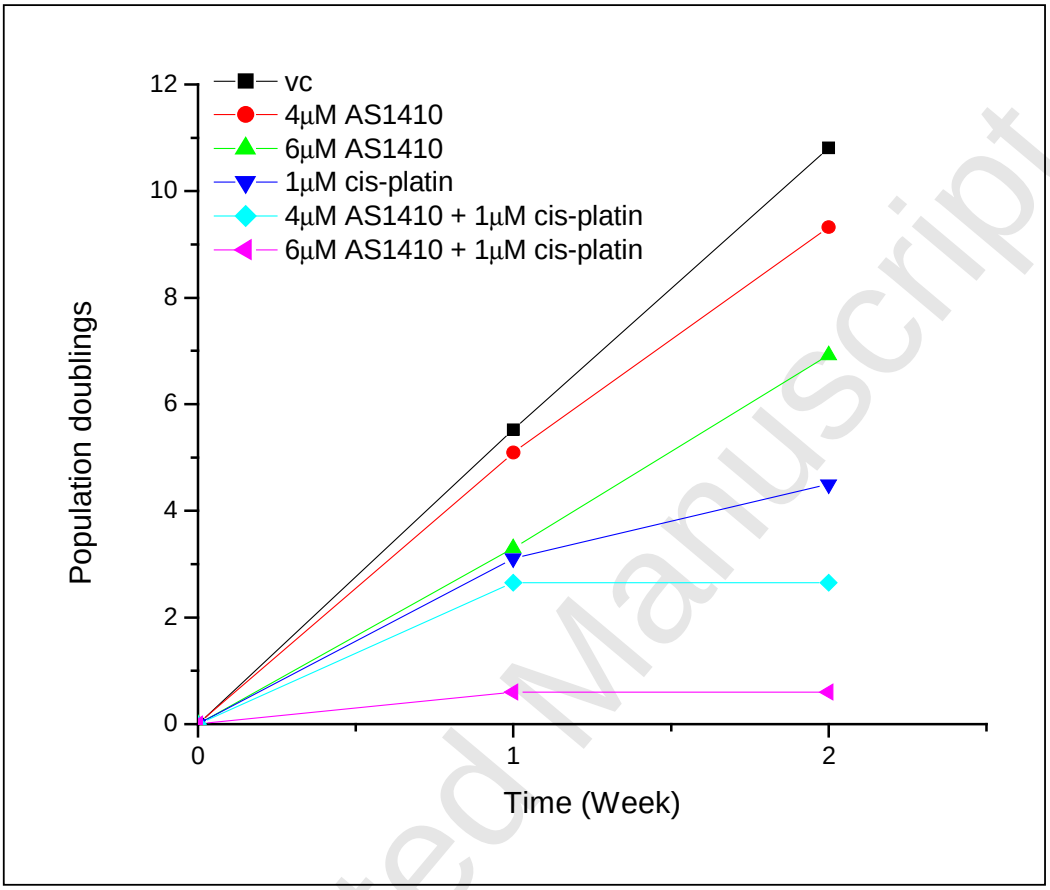

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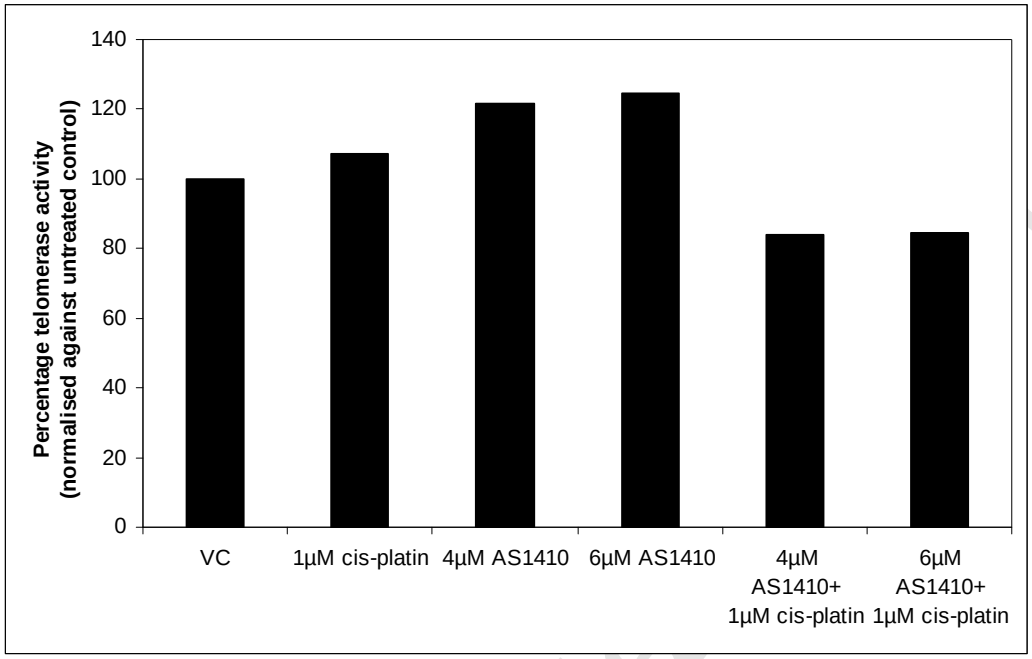

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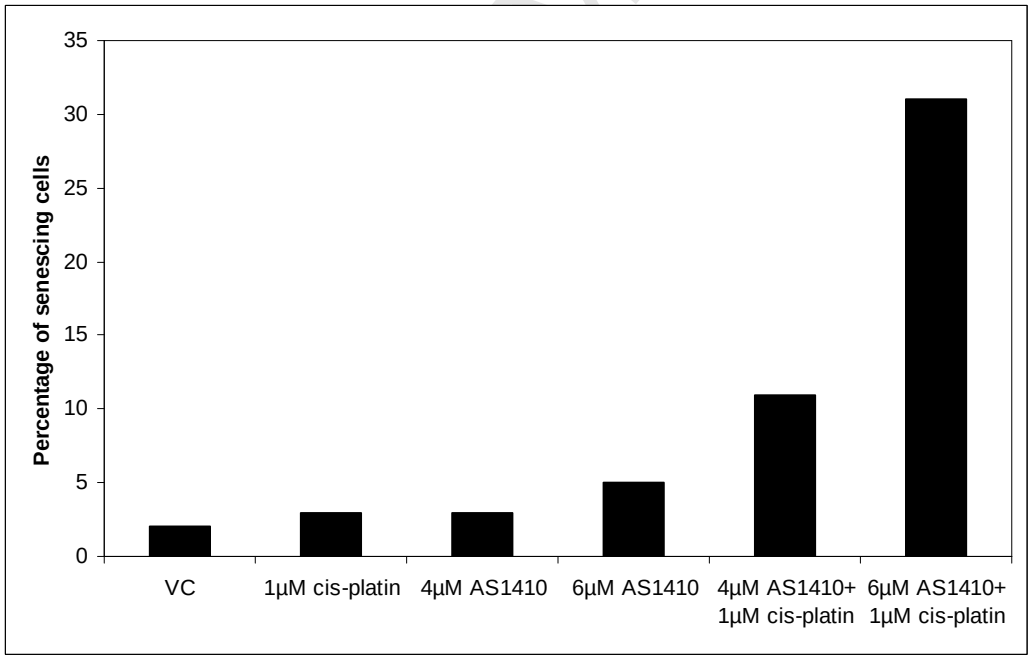

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Figure 4(c)

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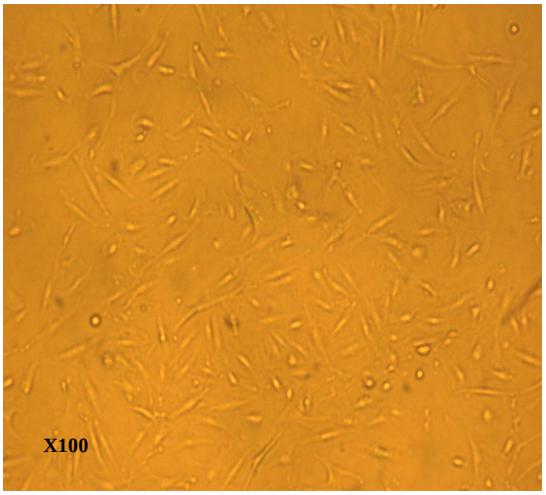

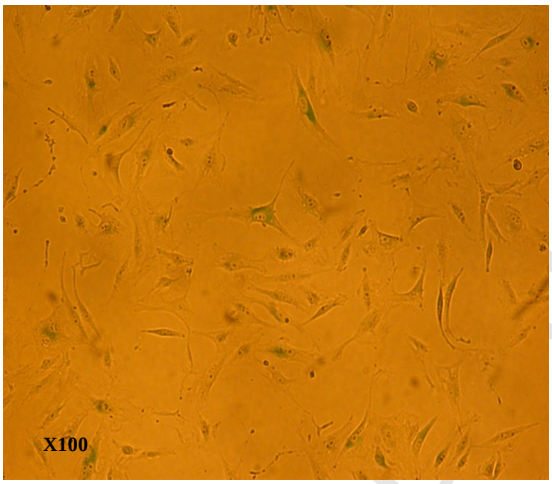

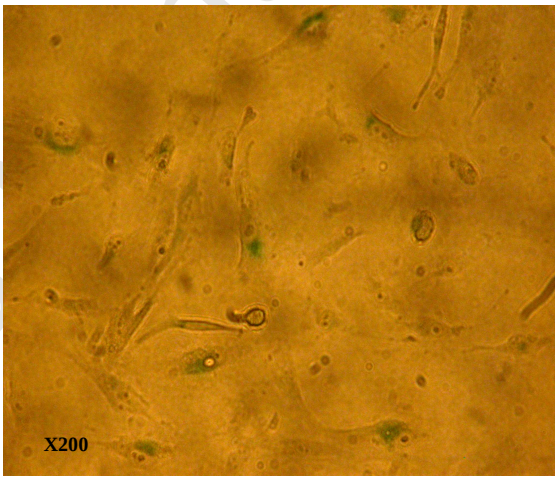

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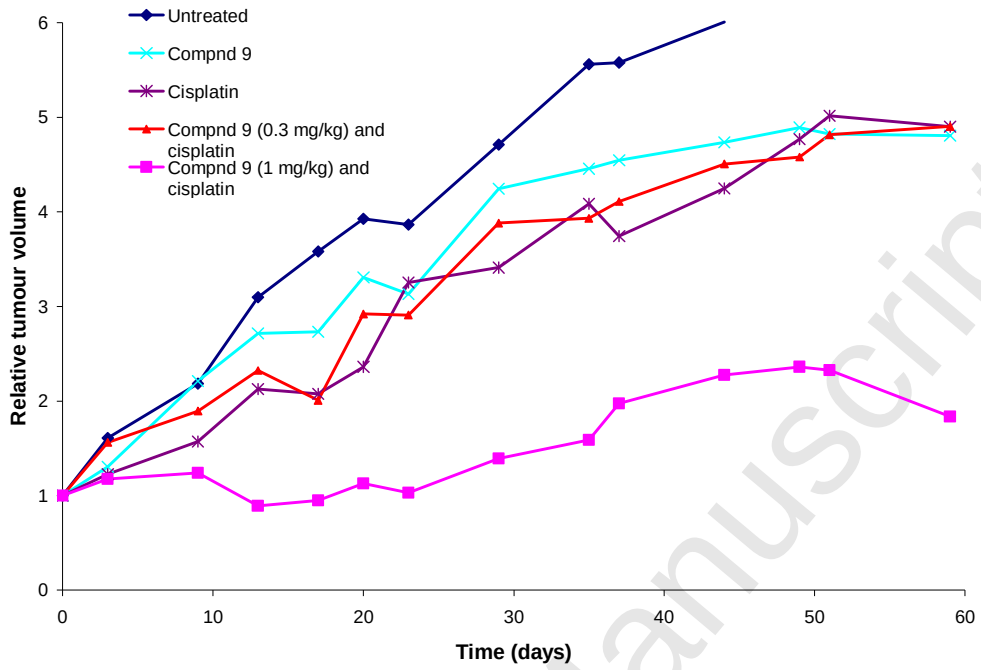
Figure 5



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