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SHORT REPORT

Therapeutic reactivation of mutant p53 protein by quinazoline derivatives

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Summary *Purpose* The human tumour suppressor protein p53 is mutated in nearly half of human tumours and most mutant proteins have single amino acid changes. Several drugs including the quinazoline derivative **1** (CP-31398) have been reported to restore p53 activity in mutant cells. The side chain of **1** contains a styryl linkage that compromises its stability and we wished to explore the activity of analogues containing more stable side chains. *Methods* Reactivation of p53 function was measured by flow cytometry as the ability to potentiate radiation-induced G_1-phase cell cycle arrest and by western blotting to determine expression of $p21^{WAF1}$. DNA binding was measured by competition with ethidium and preliminary pharmacological and xenograft studies were carried out. *Results* Screening of analogues for potentiation of radiation-induced G_1-phase cell cycle arrest using NZOV11, an ovarian tumour cell line containing a $p53^{R248Q}$ mutation, demonstrated that the (2-benzofuranyl)-quinazoline derivative **5** was among the most active of the analogues. Compound **5** showed similar effects in several other p53 mutant human tumour cell lines but not in a p53 null cell line. **5** also potentiated $p21^{WAF1}$ expression induced by radiation. DNA binding affinity was measured and found to correlate with p53 reactivation activity. Plasma concentrations of **5** in mice were sufficient to suggest in vivo activity and a small induced tumour growth delay (7 days) of NZM4 melanoma xenografts was observed. *Conclusion* Compound **5** restores p53-like function to a human tumour cells lines expressing a variety of mutant p53 proteins, thus providing a basis for the design of further new drugs.

Keywords TP53 mutation · Quinazoline synthesis · Cell cycle arrest · Flow cytometry · Tumour growth delay

Introduction

The p53 pathway plays a key role in the response of normal cells to DNA damage, hypoxia, metabolic stress and oncogene activation, leading to its description as a "guardian of the genome" and "a cellular gatekeeper" [1, 2]. In cells with wild-type p53 function, a low cellular concentration of p53 protein is maintained through a negative feedback system involving marking of proteins for proteolysis by the mdm2 ubiquitin ligase. Under conditions of cellular stress such as DNA damage or hypoxia, p53 becomes modified, leading to accumulation of protein. Consequent induction of gene transcription lead to cell cycle arrest, apoptosis and DNA repair [3]. Recent data also suggests that p53 can target mitochondria; the p53 protein interacts with multiple members of the bcl-2 family, potentially leading to a permeability transition of the outer mitochondrial membrane, apoptosis and necrosis [4]. This could explain the previously observed activation of inflammatory and vascular responses that contribute to tumour regression in a murine tumour following induction of p53 by a tetracycline transactivator protein [5].

Loss of p53 function can occur through mutation or loss of the *TP53* gene, or through altered mdm2 function. Such changes occur in a significant proportion of human cancers

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and are generally thought to be associated with poor prognosis [1, 2]. Most *TP53* gene mutations in human cancers involve single amino acid changes in the p53 protein that destabilise its conformation, leading to dominant-negative inhibition of wild-type p53 function as well as gain-of-function changes [6]. Reactivation of p53 function in cells expressing mutant p53 protein is therefore an important goal in cancer therapy.

Therapeutic strategies aimed at restoring or augmenting p53 have been a strong focus for cancer research [7]. Research on small molecule drugs that reactivate the function of mutant p53 has resulted in the identification of a number of compounds including CP-31398 (**1**; structure in Fig. 1), Prima-1 and PhiKan083 [8]. **1** was originally selected from a library of compounds and found to activate p53 function *in vitro,* as well as suppressing the growth of A375.S2 melanoma ($p53^{R249S}$) and DLD-1 colon carcinoma ($p53^{S241F}$) tumours *in vivo* [9]. Subsequent studies demonstrated that treatment with **1** reduced the incidence of UV-induced skin cancer in mice, suggesting that maintenance of p53 function could be important in preventing the development of such cancers [10, 11]. The molecular action of **1** is not yet understood but a biophysical study has suggested that **1** acts by binding to DNA rather than to p53 protein itself [12]. **1** induced p53 reporter gene activity and p21 expression in glioma cell lines with wild-type or mutant p53, but not in $p53^{null}$ cells [13] and also appeared to act in a p53-independent manner to increase expression of bax [12].

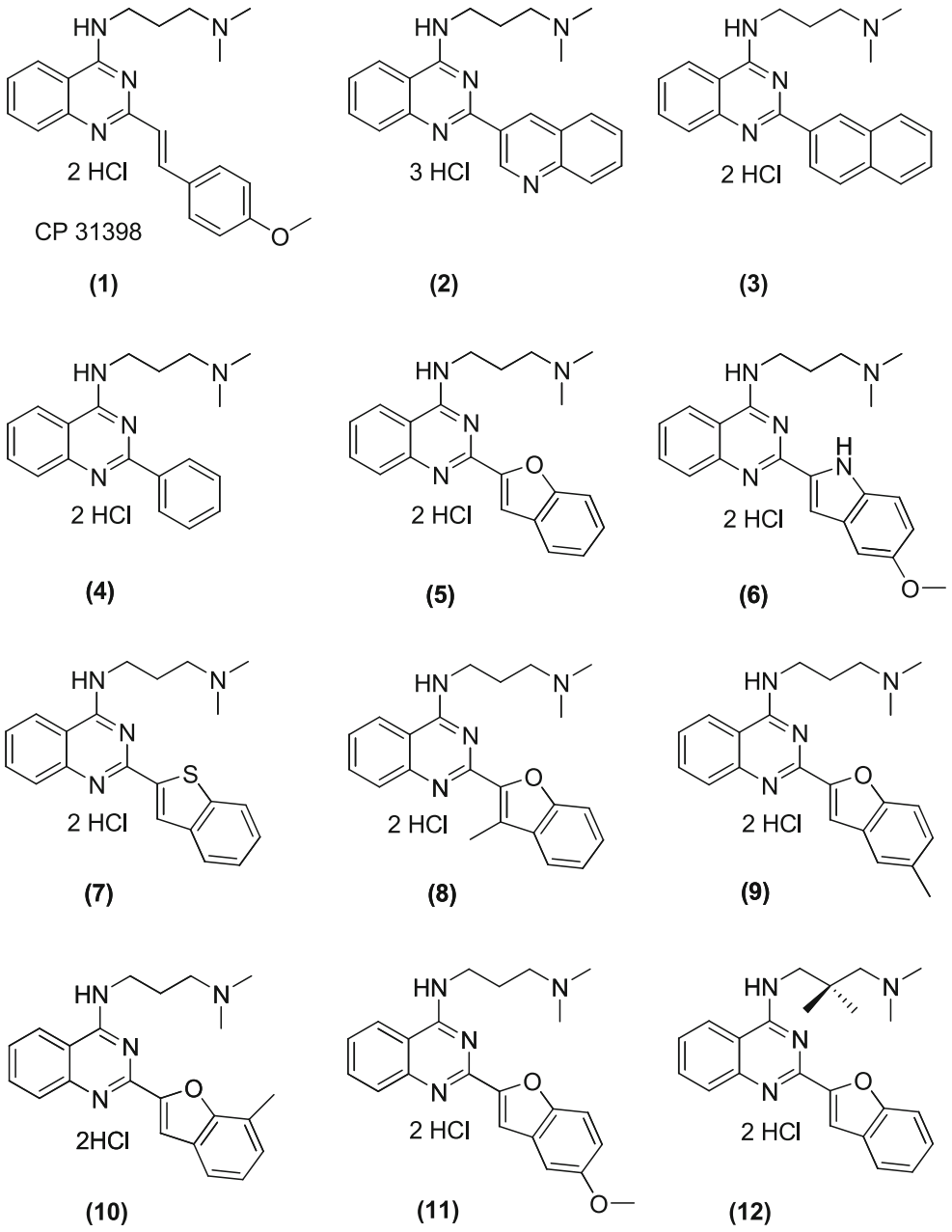

Fig. 1 Structures of the quinazoline derivatives used in this study

1 has been reported to be chemically unstable in solution [12] and the styryl linkage is the most likely component of the molecule that compromises its stability. We wished to determine whether side chain analogues of **1** lacking a styryl linkage might have equivalent activity but increased stability. Here we report the synthesis of a series of quinazoline derivatives with stable side chains and measures of their ability to reactivate p53 function using both cellular and molecular assays. Several compounds, including the (2-benzofuranyl)quinazoline derivative (**5**), were found to have p53 reactivating ability equivalent to **1** and we examined the ability of **5** to reactivate p53 function in tumour cell lines with a variety of TP53 mutations. We also measured the DNA binding constants of these analogues and compared them to their ability to reactivate mutant p53 function. Finally, we examined the antitumour potential of **5** *in vivo*.

Materials and methods

Synthesis of quinazoline analogues

The main schemes for synthesis analogues shown in Fig. 1 are indicated in Fig. 2. The styryl quinazolinone (**1a**) was synthesised by the sodium acetate mediated condensation of **13** with 4-methoxybenzaldehyde [14]. Conversion of **1a** to the intermediate chloroquinazoline and subsequent amination [15] gave **1**. The quinazolinones (**2a**–**4a**) were prepared by the Suzuki coupling of the chloroquinazolinone (**14**) with an aryl or heteroaryl boronic acid, while the quinazolinones (**5a**–**11a**) were synthesised by reaction of anthranilamide (**15**) with an aroyl chloride and subsequent cyclisation of the intermediate diamide using 5% aqueous KOH and ethanol. Conversion of the quinazolinones (**2a–11a**) to the intermediate chloroquinazolines was achieved using either thionyl chloride and dimethyl formamide, or phosphorus oxychloride and tetramethylammonium chloride. Chlorination of benzofuran substituted quinazolinones using phosphorus oxychloride instead of thionyl chloride avoided the formation of small amounts of by-products where chlorination had occurred on the benzofuran ring, separation of these by-products from the desired product generally required preparative HPLC. Reaction of the intermediate chloroquinazolines with *N*,*N*-dimethyl-1,3-propanediamine or *N*,*N*,2,2-tetramethyl-1,3-propanediamine in refluxing dioxane gave aminoquinazolines (**2**–**11**) and (**12**) respectively, which were isolated as their hydrochloride salts. Detailed methods are provided in the Supplementary Information.

Cell lines and culture conditions

Human tumour cell lines with characterised *TP53* mutations were either established in this centre [16] or were obtained through the courtesy of the US National Cancer Institute [17]. Most of the cell lines established in this centre have

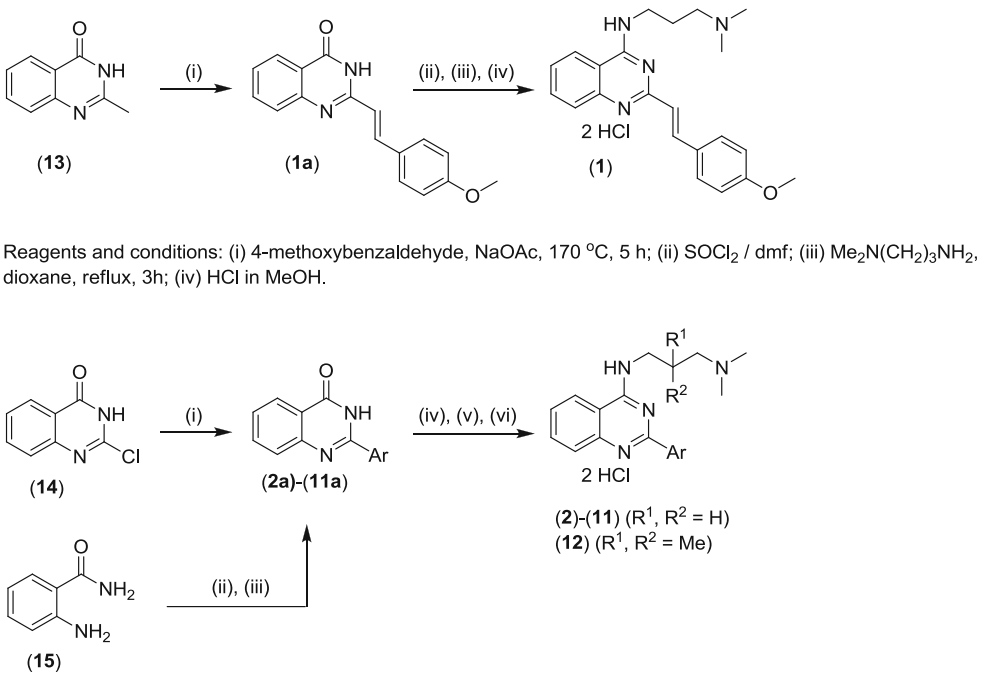

Fig. 2 Scheme for synthesis of quinazoline derivatives. See also supporting information

been deposited with CellBank Australia. HCT116 cells were kindly provided by Dr B. Vogelstein. Cell lines were cultured in α-modified minimal essential medium (α-MEM) containing insulin, transferrin, selenite, penicillin and streptomycin as previously described [17]. Growth medium was supplemented with 5% foetal bovine serum (FBS) for NZM4 and NZOV11 cells and 10% FBS for SKMEL28 and HCT116 cells. Growth medium for NZOV11 was further supplemented with epidermal growth factor and oestradiol [17].

Cell-based assay of p53 function using flow cytometry

Cells were grown in culture dishes (10^6 per dish) for at least 18 h prior to drug treatment. Unless otherwise stated the cells were radiated at 9 Gy to induce a DNA damage response and paclitaxel (200 nM) was used to inhibit cell division. Treated cells were harvested by trypsinisation, washed in PBS containing 1% FCS as blocking buffer, resuspended in ice-cold methanol and stored at −20°C. Prior to analysis, samples were washed and resuspended in PBS containing 3% FCS and treated with RNase (0.1 mg/ml, Roche Applied Science, IN, USA) and propidium iodide (0.02 mg/ml, Sigma-Aldrich) prior to analysis for DNA content on a flow cytometer (FACScan; Becton Dickinson, CA, USA).

DNA damage was estimated by γ-phosphorylation of histone H2AX. Following drug exposure, cells were harvested by trypsinisation, washed in PBS, resuspended in ice cold ethanol (70%) and stored for at least 18 h at −20°C. Prior to analysis, samples were washed in blocking buffer and incubated on a shaker platform for at least 2 h at 225 rpm. Blocking buffer was removed γ-H2AX primary antibody (200 μl, diluted 1:500 in blocking buffer), was added and cells were incubated on a shaker platform (2 h, 225 rpm). Samples were washed once in blocking buffer and incubated in secondary antibody (Alexa fluor 488) diluted in blocking buffer in the dark on a shaker platform. Cells were then washed five times, resuspended in blocking buffer (1 ml) and stored in the dark at 4°C until analysis on the same day. Ribonuclease and propidium iodide were added in the dark 10 min prior to analysis to stain cellular DNA. Fluorescence associated with γ-H2AX (green) and propidium iodide (red) was measured on a flow cytometer and results were analysed using Modfit LT software.

Determination of p53 function by western immunoblotting

Total cellular protein extracts of the treated cells were prepared using a buffer containing 10 mM Tris-HCl pH 7.5, 1 mM EGTA, 150 mM NaCl, 0.5% deoxycholate, 1% NP-40, 1 mM Na_3VO_4, 1 mM NaF, 0.1% SDS and protease inhibitor cocktail (P1860, Sigma-Aldrich, MO, USA). The resulting supernatants containing the whole cell protein extracts were collected. Protein samples (30 μg) were fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% gel) then transferred to nitrocellulose membranes. The membranes were blocked in 5% non-fat milk in 0.1% Tween 20 in PBS (blocking buffer) for 1 h and probed overnight at 4°C with anti-p21 antibody (C-19, sc-397, Santa Cruz Biotechnology) or anti-actin antibody (MAB1501R, Chemicon) in blocking buffer. Cells were washed with 0.1% Tween 20 in PBS. Goat anti-mouse or rabbit IgG horseradish peroxidise (HRP)-conjugate was applied as a secondary antibody diluted in blocking buffer (1:2000; Santa Cruz) for 1 h. Membranes were washed three times with PBS-Tween 20 and immunoreactivity was detected by subjecting the membranes to chemiluminescence detection system (ECL; Pierce Biotechnology). The signal from the membrane was detected and imaged by LAS-3000 imager (Fujifilm Corporation).

Cell proliferation (IC_{50}) assays

Cells were cultured in 96-well plates in triplicate with drug added in 2-fold serial dilutions. Cultures were incubated for 48 h and [^{3}H-methyl] labeled thymidine (20 Ci/mM, 0.04 μCi per well), together with unlabelled thymidine (TdR; 0.1 μM final concentration) and 5-fluoro-2′-deoxyuridine (FUdR; 0.1 μM final concentration) were added to each well over the last 6 h. Cells were gently dislodged with 0.5 mg/mL pronase in 2 mM EDTA (150 μL per well) at 37°C, harvested and radioactivity measured in a liquid scintillation counter (Wallac, OY, Finland).

Pharmacokinetics

Female C57 mice were treated intraperitoneally with **5** (100 mg/kg). Blood was collected from terminally anaesthetised mice 1–4 h later (3 mice per time point) in 1.5 ml microfuge tubes containing 7.5% EDTA (20 μl). Tubes were centrifuged (5,000*g*; 10 min) and plasma samples were removed and deproteinised by addition of 1 ml of ice-cold acetonitrile:water (3:1) to 100 μl plasma. Compound **9** (0.1 μM) was added as an internal standard. After mixing and centrifugation (3,000*g*; 15 min; 4°C), the supernatants were concentrated in a centrifugal vacuum concentrator, reconstituted in 100 μl mobile phase (80% acetonitrile:45 mM formate buffer (35:63), injected (5 μl) into a LUNA C18 5 μ 50×0.5 mm stainless steel (Phenomenex) and run at room temperature (initial flow rate 15 μl/min) on an Agilent capillary liquid chromatograph with ion trap detection.

Xenograft studies

Rag1 immunodeficient mice were bred and housed at constant temperature and humidity with sterile bedding and food (Vernon Jansen Unit, the University of Auckland).

All experiments were approved by the University of Auckland Animal Ethics Committee and conformed to international guidelines [18]. NZM3 cell lines were grown in culture and inoculated subcutaneously (10^7 cells/mouse) in one flank. Tumours were allowed to grow to a diameter of 4–6 mm before treatment with **5**, which was dissolved in water and injected intraperitoneally. Tumour size was measured twice weekly and tumour volumes were calculated as $0.52a^2b$, where a and b are the minor and major axes of the tumour, respectively. Tumour growth delay was determined as the difference in the number of days required for the control versus treated tumours to increase to ten times the initial volume.

Results

Development of a cellular assay for reactivation of mutant p53

An ovarian cancer cell line (NZOV11) expressing mutant p53 protein with a single amino acid change ($p53^{R248Q}$) was used to screen for activity. As shown in Fig. 3a, the DNA flow cytometry profile of untreated cells was typical of a proliferating population. Radiation (9 Gy) was used to induce a DNA damage response and paclitaxel to inhibit cell division [17, 19]. Radiation at 9 Gy followed by exposure to paclitaxel for 24 h caused movement of most G_1-phase cells to G_2/M-phase, with no cells dividing and a small proportion entering another cell cycle without dividing (Fig. 3b). Addition of **1** together with radiation and paclitaxel, followed by culture for 24 h, increased the proportion of cells that remained in G_1-phase; this proportion was dependent on the concentration of **1** (Fig. 3c–e). Addition of **1** alone to untreated cultures did not increase the proportion of G_1- or G_2-phase cells (data not shown).

Screening of quinazoline derivatives in a cell-based assay

Derivatives with differing side chains at the 2-position (Fig. 2) were synthesised and tested in the above assay using NZOV11 at concentrations of 2.2, 6.7 and 20 μM. An advantage of the cell-based assay system was that other drug effects on cell growth, including the induction of cell death, could also be monitored by flow cytometry. The results are shown in Fig. 4. Compounds **5** and **7** were the

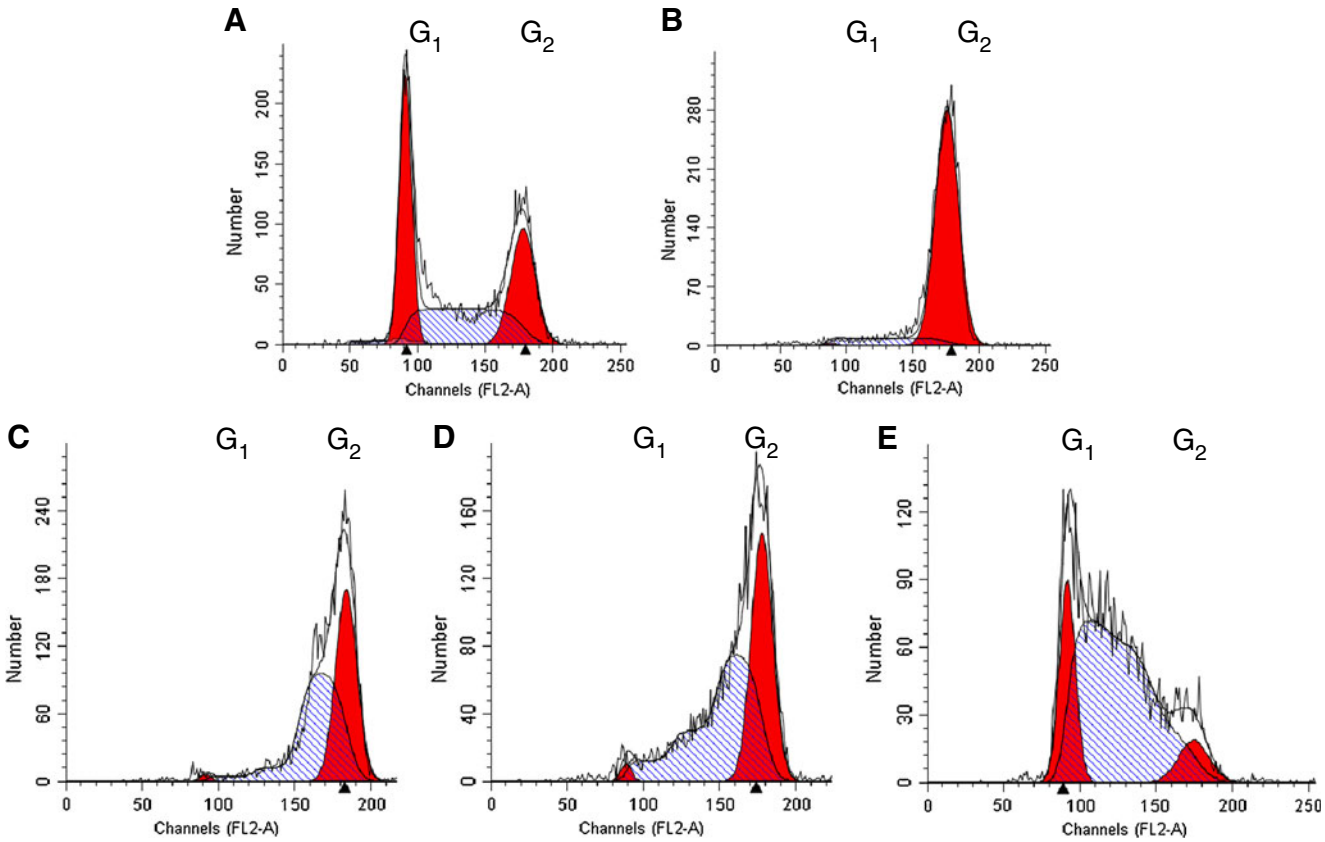

Fig. 3 Flow cytometry profiles of NZOV11. Red fluorescence from propidium staining is used as an indicator of DNA content (positions of G_1- and G_2-phase cell populations are marked). **a** Untreated cells. B. Irradiation at 9 Gy followed by culture in the presence of paclitaxel (200 nM) for 24 h, showing induction of G_2/M-phase arrest. C, D, E. Accumulation of cells in G_1- and S-phases following addition of 1 at concentrations of 2.2, 6.7 and 20 μM, respectively, in addition to irradiation and addition of paclitaxel

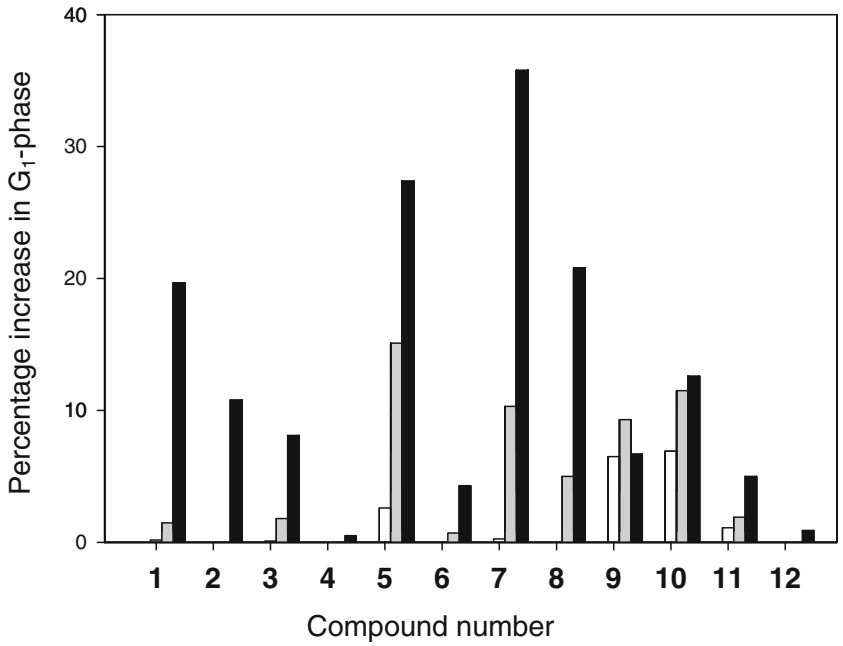

Fig. 4 Comparison of the ability of quinazoline derivatives at concentrations of 2.2 μM (*white bars*), 6.7 μM (*gray bars*) and 20 μM (*black bars*) to retain NZOV11 cells in G_1-phase 24 h after irradiation (9 Gy) and addition of paclitaxel (200 nM)

most effective of the analogues in the ability to induce retention of cells in G_1-phase. Compound **5** was selected for further study.

Induction of p53 transcription products

Since $p21^{WAF1}$, a transcription product of p53, is thought to be responsible for the induction of G_1-phase arrest, its cellular concentration was measured by western blotting. As shown in Fig. 5, both **1** and **5** induced p21 expression in NZOV11 cells in a concentration-dependent manner. Increases in p21 paralleled increases in the proportion of G_1-phase cells measured by flow cytometry.

Effects of compound 5 on other p53 mutant lines

We next determined whether **5** could reactivate p53 in cell lines expressing other mutant p53 proteins. Because cell lines grew at different rates, the proportion of G_1-phase cells in the negative controls (i.e. treated with radiation and paclitaxel alone) varied among the cell lines, and the results were scored as percentage increases in G_1-phase content. As shown in Table 1, **5** caused an increase in G_1-phase content in all TP53 mutant cell lines tested except for the line that had a frameshift mutation. A similar experiment using HCT116 $p53^{-/-}$ cells showed that **5** did not induce G_1-phase arrest, also suggesting that the effect was p53-dependent.

Effects of compound 5 on NZOV11 cells in the absence of radiation

Addition of **5** in the presence of paclitaxel but without irradiation, followed by culture for 24 h, also increased the proportion of G_1-phase cells in a dose-dependent manner, although the effects were smaller than those observed in the presence of radiation (Fig. 6b). Addition of **1** under similar conditions also increased the proportion of G_1-phase cells in a dose dependent manner (data not shown).

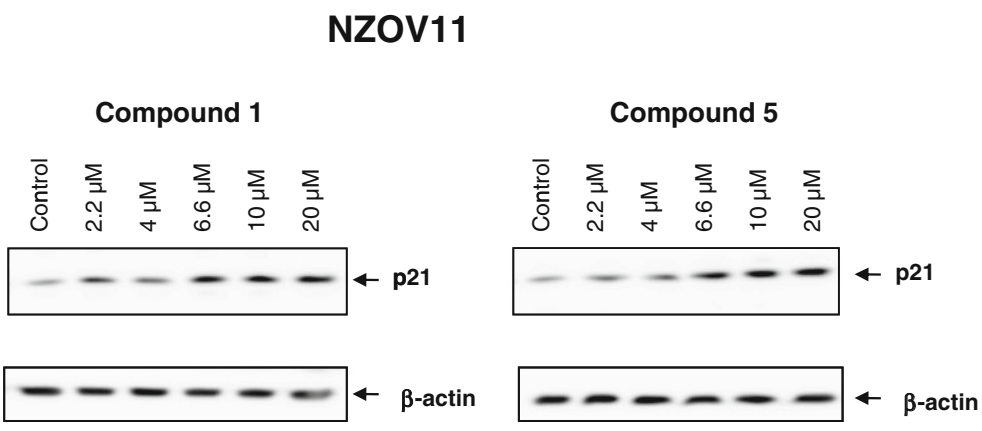

Fig. 5 Expression of p21 in NZOV11 cells treated for 24 h with **1** and **5**, at the indicated concentrations, after irradiation at 9 Gy. The loading control was β-actin

Table 1 Properties of cell lines used in this study and their responses to **5**. Cells in each case were irradiated to 9 Gy and grown in the presence of paclitaxel (200 nM) **5** (20 μM) for 24 h before measurement of G_1-phase cell content

Cell line	Tumour type	p53 status	Induced G_1-arrest %
NZM4	Melanoma	$p53^{S241T}$	50
NZM7	Melanoma	$p53^{S241T}$	22
SKMEL2	Melanoma	$p53^{G245S}$	20
SKMEL28	Melanoma	$p53^{C145V}$	26
NZOV1	Ovarian	$p53^{H178T}$	16
NZOV2	Ovarian	$p53^{Y220D}$	14
NZOV8	Ovarian	$p53^{S215G}$	17
NZOV10	Ovarian	$p53^{Y220F}$	11
OVCAR3	Ovarian	$p53^{R248Q}$	7
OVCAR8	Ovarian	$p53^{Y126\_K132del}$	0
SKOV3	Ovarian	$p53^{H179R}$	4
T47D	Breast	$p53^{L194F}$	17
HT29	Colorectal	$p53^{R273H}$	5
SW620	Colon	$p53^{R273H}$	2
HCT116 $p53^{-/-}$	Colon	$p53^{null}$	0

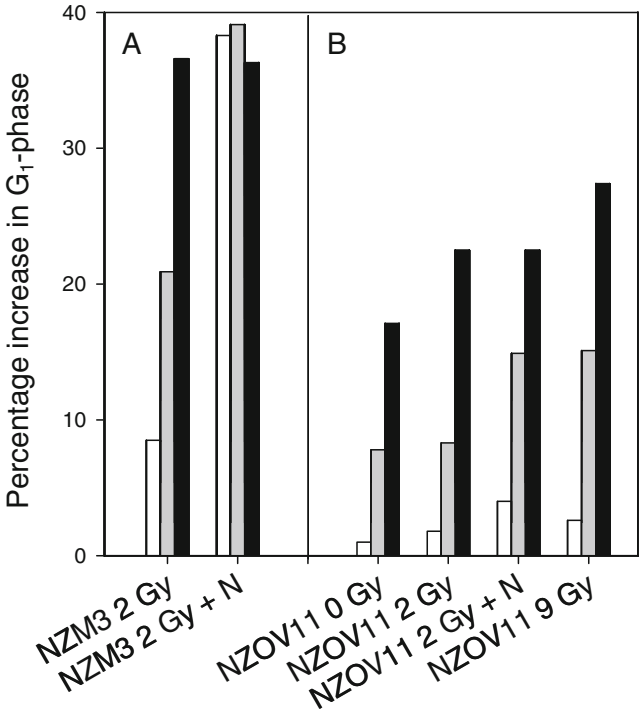

Fig. 6 **a** Comparison of the ability of **5** at concentrations of 2.2 μM (*white bars*), 6.7 μM (*gray bars*) and 20 μM (*black bars*) to retain NZM3 melanoma cells (p53 wild-type) in G_1-phase 24 h after radiation (2 Gy) and addition of paclitaxel (200 nM). The effect of adding nutlin-3 (10 μM) to the cultures is indicated by the label (+N). **b** Comparison of the ability of **5** at concentrations of 2.2 μM (*white bars*), 6.7 μM (*gray bars*) and 20 μM (*black bars*) to retain NZOV11 ovarian cancer cells (p53 mutant) in G_1-phase 24 h after after radiation (2 Gy) and addition of paclitaxel (200 nM). The effect of adding nutlin-3 (10 μM) to the cultures is indicated by the label (+N)

Effects of compound **5** on cells expressing wild-type p53

One interpretation of the above results is that **5** stabilises p53 in an active conformation, leading to increased downstream effects. If this were the case, **5** might also stabilise wild-type p53, leading to enhanced p53 responses. Cell cycle responses were therefore measured using the NZM3 melanoma line, which expresses wild-type p53 protein [16]. Because radiation at 9 Gy alone induced a high degree of G_1-phase arrest (45%) in this cell line, a lower radiation dose (2 Gy) was selected. Radiation and paclitaxel alone resulted in 8.5% G_1-phase content, and addition of **5** induced a dose-dependent increase in G_1-phase content (Fig. 6a), consistent with an effect on wild-type p53 function. The drug nutlin-3 is known to enhance the activity of wild-type p53 expression by inhibiting mdm2-mediated degradation of p53 [20], and its effect was investigated. At a concentration of 10 μM, nutlin alone induced a high degree of G_1-phase arrest (36%), consistent with its enhancement of a p53 response to DNA damage. Addition of **5** as well as nutlin did not induce further arrest (Fig. 6a).

The results of previous sections suggests that **5** stabilises mutant p53, and if this then behaved like wild-type p53 protein one might expect nutlin-3 to further enhance its stability. The effect of addition of nutlin-3 in addition to **5** on the NZOV11 p53 mutant cell line was therefore examined. Following radiation at 2 Gy and growth in paclitaxel, the proportion of G_1-phase cells in NZM3 cells after 24 h was found to be 6% with or without the addition of nutlin-3 at the time of irradiation (data not shown). The effect of **5** was most evident at a concentration of 6.7 μM (Fig. 6b). The proportion of G_1-phase cells in NZM11 cells was 8% in the absence of nutlin-3, but the addition of nutlin-3 (10 μM) increased the degree of G_1-phase arrest induced by **5** to 15% (Fig. 6b), suggesting increased p53 activity.

Relationship between p53 reversion activity and DNA binding

Since **1** is reported to bind DNA [12], it is possible that its effects are mediated by DNA. To determine whether DNA binding affinity was related to the biological effects of the quinazoline derivatives, DNA binding was measured by competition with ethidium as previously described [21]. DNA binding affinity for both poly(dAT).poly(dAT) was found to correlate (r=0.84; p<0.01) with the induction of p53 G_1-phase arrest in the cell-based assay (Fig. 7). A

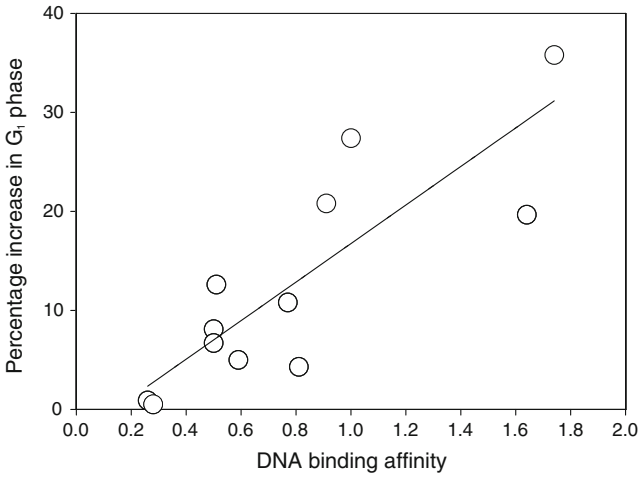

Fig. 7 Relationship between DNA binding affinity and p53 reactivation. DNA binding was measured by competition with propidium iodide for binding sites on the double-stranded synthetic DNA poly (dAT).poly(dAT)

slightly lower correlation with binding to poly(dGC).poly (dGC) was also observed (data not shown).

Relationship between p53 reversion activity and topoisomerase II poisoning

Many DNA binding drugs, particularly acridine derivatives, interact with the enzyme topoisomerase II and induce DNA damage in the form of double-stranded breaks, with consequent cytotoxicity [22]. Furthermore, several active analogues of **1** are acridine derivatives [9]. The DNA binding ability of **5** raised the question of whether it also induced DNA damage. The DNA damage response of NZOV11 cells was assessed by measuring γ-phosphorylation of histone H2AX by two dimensional flow cytometry, with propidium staining of DNA in the second dimension (Fig. 8). As a positive control, the topoisomerase II poison amsacrine [23] was tested. H2AX phosphorylation was evident in the case of amsacrine but not with **5** or **1**, which instead each induced depletion of S-phase cells. The activity of **1** and **5** as poisons of topoisomerase II was also tested utilising Jurkat, a TP53 null human leukaemia cell line, together with two derived cell sub-lines (JL_A and JL_D) that had been selected for resistance to amsacrine and doxorubicin, respectively [24] and expressed low amounts of topoisomerase II protein [25]. As shown in Table 2, neither line was cross-resistant to **1** or **5**, consistent with the hypothesis that they are not topoisomerase poisons. It was of interest that **5** showed slightly greater activity against the resistant sub-lines than against the parental line, but the reason for this was not investigated.

In vivo activity of **5**

We determined whether plasma concentrations of **5** that were active in reactivating p53 could be achieved *in vivo*. An analytical procedure for the determination of **5** concentrations in mouse plasma was developed using high performance liquid chromatography and ion trap mass spectrometry. **5** was well tolerated in C57Bl6 mice with a maximum tolerated daily intraperitoneal dose of 100 mg/kg. The maximum plasma concentration of 8.5 μM (Fig. 9) was in the range of concentrations found to be active in the cell-based assays. We next investigated the ability of **5** to affect the growth of the NZM4 human melanoma line ($p53^{S241T}$) as a xenograft growing in immunodeficient mice. Treatment with **5** alone (100 mg/kg) had no effect on tumour growth while radiation (2 Gy) resulted in a growth delay of 1.5 days. Administration of radiation (2 Gy) and **5** in combination resulted in a small tumour growth delay of 7 days (Fig. 9).

Discussion

A cell-based assay measuring the potential of drugs to potentiate the induction of G_1-phase arrest in a human p53 mutant cell line was used to evaluate a series of quinazoline derivatives related to **1**. This screen identified several analogues of **1** that lacked the relatively unstable styryl

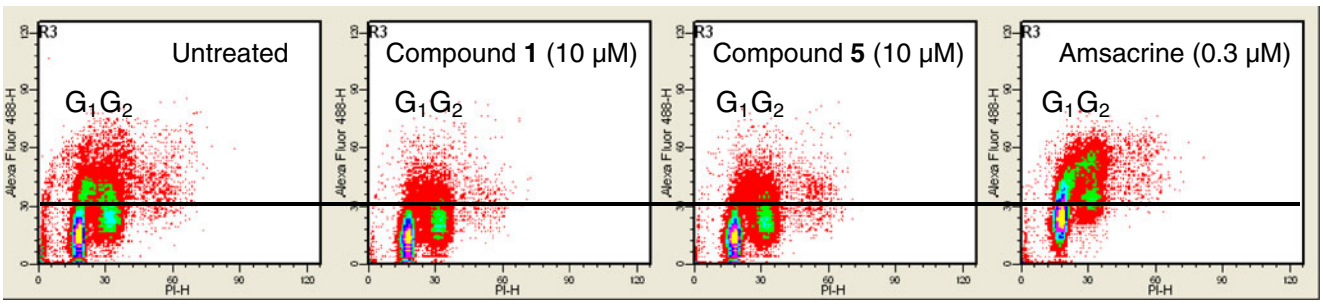

Fig. 8 Flow cytometry profiles of NZOV11 cells either untreated or exposed to **1**, **5** or amsacrine at the indicated concentrations. The *x*-axis indicates red fluorescence from propidium staining as an indicator of DNA content (positions of G_1- and G_2-phase cell populations are marked). The *y*-axis indicates green staining from antibody to phosphorylated histone H2AX as a measure of DNA damage

Table 2 Growth inhibitory concentrations of **1** and **5** on the Jurkat human leukaemia cell line (JL) and on two sub-lines (JL_A and JL_D) that express low amounts of topoisomerase II [25]

IC_{50} values (μM)	JL	JL_A	JL_D
1	3.5	3.0	2.0
5	8.0	5.4	5.5
Amsacrine	0.027	0.26	0.30
Doxorubicin	0.007	0.028	0.112

side chain but retained comparable activity (Fig. 4). Some structure-activity relationships can be gleaned from these data. The poor activity of **2**, **3**, **4** and **6** attest to a clear preference for benzofuran or benzothiophene side chains, which place the phenyl group of the aromatic side chain in approximately the same position as that of **1**. Compounds **7** and **5** were the most active with the former showing more activity at 20 μM and the latter more activity at 6.7 μM (Fig. 3). The activity of compounds **8**–**11** suggested minimal bulk tolerance around the benzofuran while lack of activity of **12** suggested reduced tolerance around the aliphatic 3-(dimethylamino)propyl side chain.

Compound **5** was investigated further and shown to be capable of potentiating G_1-phase arrest in several cell lines containing different *TP53* mutations, but not in a *TP53* null line or in a line containing a frameshift mutation of the *TP53* gene (Table 1). Addition of **1** or **5** to NZOV11 cells increased expression of $p21^{WAF1}$, a cyclin-dependent kinase inhibitor product of a p53-target gene, consistent with p53 mediation of G_1-phase arrest. Taken together with previous data for **1**, the results suggest that **5** acts to restore the function of a variety of mutant p53 proteins. The cell-based screen provides a rapid means of assessing the ability of drugs to reactivate mutant p53 function and suggests that other active, chemically stable analogues of **5** may be found.

Since the aim of this study was to identify drugs that potentiated a p53 responses to radiation-induced DNA damage, it was surprising to find in a control experiments that addition of **5** or **1** to cultures in the absence of ionising radiation also induced G_1-phase arrest, although of smaller magnitude than when used in combination with radiation at 2 Gy or 9 Gy (Fig. 6). A likely explanation for this effect is that **5** potentiates the p53 response to endogenous DNA damage; many tumour cell lines have short telomeres or other chromosomal characteristics that induce an endogenous DNA damage response [26]. The positive relationship between induced G_1-phase arrest and DNA binding affinity (Fig. 7), together with the known ability of many DNA binding drugs to induce DNA damage through effects on the enzyme topoisomerase II [27], raises the question of whether these drugs themselves induce DNA damage as part of their cellular cycle effects. However, phosphorylation of histone H2AX, a standard indicator of DNA damage, was not induced by **5** or **1** but was induced by the DNA binding topoisomerase II poison amsacrine (Fig. 8).

As shown in Table 1, **5** potentiated the p53 response to radiation in a variety of cell lines with differing mutations in the *TP53* gene, but not in a cell line lacking the *TP53* gene. Unexpectedly, **5** also potentiated the p53 response to

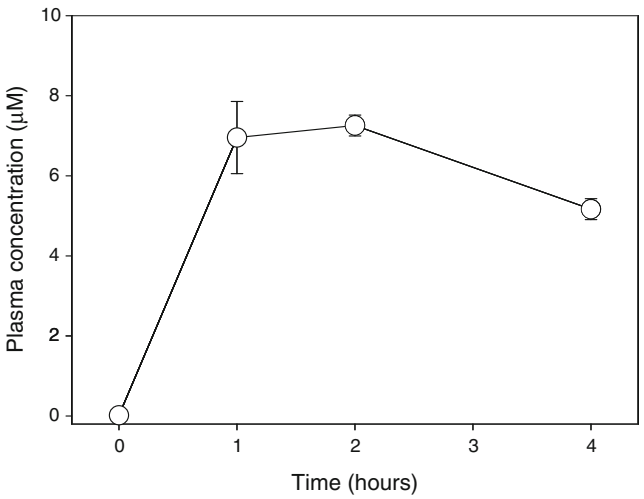

Fig. 9 Plasma pharmacokinetics of **5** in mice following a single i.p. injection (100 mg/kg; female C57BL mice)

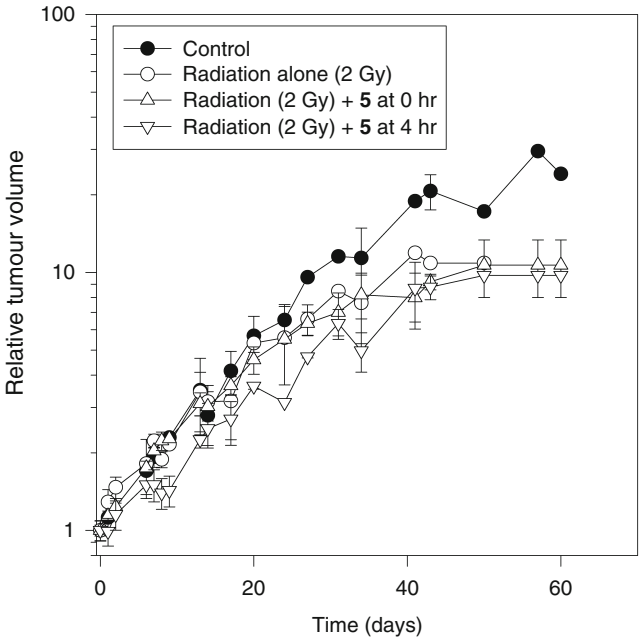

Fig. 10 Effect of radiation and **5** on the growth of NZM4 melanoma xenografts in immunodeficient mice. Mice were either untreated (•), treated with radiation at 2 Gy (○), treated with radiation plus **5** (100 mg/kg) simultaneously (Δ) or treated with radiation and 4 h later with **5** (100 mg/kg) (∇)

radiation in a cell line (NZM3) that expresses wild-type p53 protein (Fig. 4). However, these observations are all consistent with the hypothesis that **1** and **5** stabilise active conformations of both wild-type and mutant p53 proteins, allowing them to bind more productively to consensus p53 binding sites. Wild-type p53 protein, although more stable than mutant p53 proteins, is still intrinsically unstable, particularly in the presence of mdm2 [28]. These observations are also consistent with reports **1** potentiates the effects of both wild-type and mutant p53 [29]. The cytoplasmic form of the wild-type p53 protein is normally maintained in a tetrameric form with all four subunits geometrically equivalent [30] but in response to stress signals the protein is modified and the p53 proteins then bind as a linear tetramer ("dimer of dimers") to consecutive pairs of half-promoter sites on the DNA [31].

The receptor for **1** is not known and previous studies have failed to detect a physical interaction between **1** and the p53 core domain [12]. **1** binds to DNA [12] and the positive relationship between DNA binding affinity and p53 reactivation of these analogues (Fig. 7) suggests that DNA binding could be involved in the action of these compounds. Alternatively, the drugs could stabilise the p53 protein indirectly by interaction with proteins that maintain an active p53 conformation. Further work is in progress to investigate these relationships using a larger group of compounds.

Following administration of **5** to mice at a non-toxic dose, plasma drug concentrations were found to be in the same range as those causing the induction of G_1-phase arrest in the cell-based system (Figs. 4 and 9), suggesting that the drug could have *in vivo* activity. A preliminary study of the effect of **5** on a tumour xenograft of the p53 mutant melanoma line NZM3 suggested the presence of a low level of activity (Fig. 10). The original demonstration of *in vivo* activity of **1** was carried out in the absence of an added DNA damaging agent [9] but in another experiment (not shown) **5** was shown to lack activity against NZM3 cells in the absence of radiation.

In conclusion, this study has provided examples of new small molecule drugs with good chemical stability and the ability to reactivate mutant p53. The flow cytometry approach has an advantage in screening procedures because it allows estimation of both induced G_1-phase and of other induced cellular changes including the induction of apoptosis. The p53 protein has a complex action that includes effects on both gene transcription and mitochondrial function [3, 4] and reactivation by small molecule drugs remains as an important goal of medicinal chemistry. More than a decade has passed since the publication of the first drugs that can reactivate mutant p53 and although many candidate molecules have been described, clinical efficacy has not yet been demonstrated [7]. Further research may require integrated combination of molecular, cellular and whole animal studies.

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