The kinase inhibitor O\textsuperscript{6}-cyclohexylmethylguanine (NU2058) potentiates the cytotoxicity of cisplatin by mechanisms that are independent of its effect upon CDK2

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1. Introduction

Platinum compounds such as cisplatin and carboplatin remain amongst the most widely used chemotherapeutic drugs in the treatment many tumours types, including head and neck, testicular, lung, and ovarian carcinomas. For example, the combination of bleomycin, etoposide and cisplatin is considered curative therapy for testicular cancer, with survival rates of over 95% [1]. Like most cytotoxic anti-cancer drugs, cisplatin acts via the induction of DNA damage involving a number of types of DNA adducts [2].

Although cisplatin has clinical utility against some tumour types it has little activity against others. Furthermore, initially sensitive tumours can become resistant to platinum complexes. Acquired as well as intrinsic cisplatin resistance can be caused by several mechanisms, including altered drug uptake, increased activity of cellular detoxification systems and DNA repair [3–8]. Modulation of cellular responses to established drugs such as cisplatin have the potential to both improve efficacy and attenuate toxicity.

Protein kinases are involved in a wide range of cellular events including cell growth, DNA repair, replication and cell survival. Many protein kinases are deregulated in the development and progression of cancer, and protein kinase inhibitors are currently being used therapeutically to exploit the molecular pathology of...
neoplasms. Trastuzumab and imatinib are two clinically successful anti-cancer drugs which act via inhibition of kinase signalling pathways [9,10]. Encouraged by the success of imatinib and trastuzumab, the identification of protein kinases that are deregulated in cancer and the development of kinase inhibitors is currently a major drug discovery priority.

Cyclin-dependent kinases (CDK), and their partner cyclins, are key proteins in the regulation of the cell cycle and are frequently deregulated in cancer cells [11]. The CDKs have been investigated as targets for cancer therapy and the antitumour activity of CDK inhibitors has been demonstrated in preclinical models, both in vitro and in vivo [12]. However, CDK inhibitors have so far shown little or no single agent clinical activity, with the possible exception of flavopiridol for the treatment of chronic lymphocytic leukaemia [13]. These latter data are consistent with preclinical studies, where inhibition of CDKs by flavopiridol was shown to induce cell death [14]. It has also been suggested that CDK inhibitors could be used to increase the efficacy of cytotoxic therapies by modulating cellular responses, possibly by recruiting or holding cells in a drug sensitive phase of the cell cycle [15].

\(O^2\)-Cyclohexylmethylguanine (NU2058, Fig. 1A) is a compound that was identified in a programme to develop potent and selective inhibitors of CDK2. NU2058 showed modest inhibition of CDK2 with an IC\(_{50}\) (concentration required to give 50% inhibition of CDK2 in an isolated enzyme assay) value of 17 \(\mu\)M [16]. NU2058 has previously been shown to potentiate the cytotoxicity of cisplatin in vitro [17] and it was hypothesised that inhibition of CDK2 was responsible for this effect.

In the present work, we tested the hypothesis that CDK2 inhibition was responsible for the potentiation of cisplatin cytotoxicity by NU2058 through the use of two structurally related compounds; NU6102 and NU6230 (Fig. 1A). Furthermore, to improve our understanding of the mechanism of potentiation of cisplatin by NU2058 we also investigated: (1) whether NU2058 affected cisplatin–DNA interactions and cellular drug uptake; (2) if NU2058 potentiated the cytotoxicity of other DNA damaging agents; (3) whether the effect of NU2058 on potentiation of cisplatin was schedule dependent.

2. Methods

2.1. Materials

All materials were purchased from Sigma–Aldrich (UK) unless otherwise stated. Monohydroxymelphalan was synthesised according to Tilby et al. [18]. Temozolomide was provided by the Cancer Research UK Formulation Unit, Strathclyde University, UK. Cells were irradiated using X-rays (310 kV, dose rate 3 Gy/min). NU2058, NU6230, NU6102 were synthesised as described previously [16,19] and stored as 100 mM stock solutions in DMSO at \(-20^\circ\)C.

2.2. Cell lines

The human head and neck cancer cell line, SQ20b, was kindly provided by Dr. Michael Beckett (Department of Radiation and Cellular Oncology, University of Chicago, USA) and maintained in DMEM: Ham's F-12 medium, supplemented with 20% (v/v) foetal calf serum, and 0.4 mg/ml hydrocortisone, as previously described [20]. Cells were grown in the presence of penicillin (100 unit/ml) and streptomycin (100 \(\mu\)g/ml) at 37 \(\pm\) 5% CO\(_2\).

2.3. Colony formation assay

Cells were seeded into six-well plates (350,000 cells/well) and allowed to attach overnight. On the day of the experiment, growth media were replaced with media containing 100 \(\mu\)M of either NU2058, NU6102, NU6230 or DMSO (0.1% (v/v) final concentration) for 2 h followed by a further 2 h in the additional presence of cytotoxic drugs, unless otherwise indicated. For the radiation experiments, cells were treated for a total of 4 h with NU2058, and irradiated after the first 2 h. After treatment, the cells were washed twice with PBS, trypsinised, and replated into 100 mm Petri dishes at various cell densities (300–50,000 cells per plate). After approximately 12 days, media was removed, and cells were fixed with Carnoy's reagent (75% (v/v) methanol, 25% (v/v) acetic acid), stained with crystal violet (0.4% (w/v) in water) and colonies were

Fig. 1. (A) Structures of NU2058, NU6102, and NU6230 and CDK2 IC\(_{50}\) (B) Effect of NU2058, NU6102 and NU6230 on the cytotoxicity of cisplatin. SQ20b cells were pre-treated with each compound alone (100 \(\mu\)M) or vehicle (0.1% (v/v) DMSO) for 2 h and for a further 2 h in combination with cisplatin. After treatment cells were assessed for their colony forming ability after 12 days. Points are the mean, and bars are the standard deviation of three separate experiments. (C) Effect of compounds on phosphorylation of retinoblastoma protein. Cells were treated with each compound (100 \(\mu\)M) for 4 or 24 h before analysis by Western blotting for T\(_{821}\)-phospho-Rb. Results from three independent experiments are presented.
2.4. Western blotting

Phosphorylation of retinoblastoma protein (Rb) was measured in cells after incubation with each compound or DMSO. Exponentially growing cells were treated in 15 cm dishes with 100 μM of either NU2058, NU6230, NU6102 or vehicle (0.1% (v/v) DMSO) for a period of 4 or 24 h. After treatment, cells were washed twice with ice-cold PBS, and lysed with Laemmli buffer [21]. Samples were sonicated and protein concentration quantified using BCA protein assay (Pierce, USA). Equal amounts of protein (15 μg) were separated on a 4–20% (w/v) Tris–glycine SDS polyacrylamide gel (Invitrogen, UK) and transferred to a PVDF blotting membrane (BioRad, USA). The membrane was blocked in 5% (w/v) milk in Tris buffered saline containing 0.2% (v/v) Tween (TBST), and the primary monoclonal phosphospecific (pRb-T821) antibody (Biosource, CA, Cat. No. 44-582G) was added at a dilution factor of 1:1000 for 2 h, washed with TBST, and incubated with a rabbit secondary antibody (DakoCytomation, Denmark, Cat. No. P0217) in TBST containing 5% (w/v) milk for a further 2 h. The ECL system (Pierce, USA) was used for detection of phospho-Rb. The image was captured with photographic film and developed. For measurement of actin, membranes were incubated as above but with a primary anti-actin (Sigma, UK, Cat. No. A4700) and with a rabbit anti-mouse secondary antibody (DakoCytomation, Denmark, Cat. No. P0260).

2.5. Measurement of total cellular platinum and platinum–DNA adducts

Actively growing cells were treated with NU2058 (100 μM) or DMSO control for 2 h and then a further 2 h in the additional presence of cisplatin (0–50 μM). After treatment, cells were either washed twice with ice-cold PBS, trypsinised and collected by centrifugation, or washed twice with warm growth medium and further incubated at 37 °C for periods up to 24 h. For platinum–DNA adduct analysis, DNA was extracted from cells using the Qiagen DNeasy kit according to the manufacturer’s instructions (Qiagen, UK). Isolated DNA was quantified by measuring OD260 using the ND-1000 Spectrophotometer (NanoDrop, USA). Extracted DNA was hydrolysed with 3.5% nitric acid (v/v) Spectrosol grade (Merck, UK) overnight at 70 °C, prior to analysis by inductively coupled plasma mass spectroscopy (ICP-MS). For measurement of total cellular platinum, cell pellets were sonicated and protein measured as described above. Samples were then incubated with 1 M NaOH for 1 h at 60 °C, followed by an overnight acid hydrolysis with 1 M nitric acid at 70 °C. Platinum concentrations in samples were determined by ICP-MS as described previously [22], and compared to a standard curve analysed on the same day to correct for any instrument drift.

2.6. Measurement of melphalan–DNA adducts

For measurement of melphalan–DNA adducts, cells were pre-treated with 100 μM NU2058 or vehicle (0.1% (v/v) DMSO) for 2 h followed by a further 2 h in the additional presence of melphalan (0–50 μM). After drug treatment, cells were washed twice with ice-cold PBS and released using trypsin. Cells were then centrifuged and washed once more with PBS, and pellets were stored at −20 °C until required. DNA was extracted using the phenol hydroxyapatite method as described by Tilby et al. [18]. DNA concentration was determined by measuring OD260 using a ND-1000 Spectrophotometer (NanoDrop, USA). Melphalan–DNA adducts were quantified using a competitive ELISA as described previously [23]. Briefly, several dilutions of DNA from treated cells were mixed with a constant amount of MP5/73 antibody, which was specific for melphanal–guanine adducts on DNA, and incubated in wells coated with a standard amount of melphalan-treated DNA. The amount of antibody that bound to the wells was determined by a fluorogenic enzyme assay. The quantities of adducts per assay well that caused a 50% reduction of the assay signal were determined by a curve fitting procedure.

3. Results

3.1. NU2058 did not markedly inhibit CDK2 activity in vitro

The hypothesis that NU2058-enhanced cisplatin cytotoxicity is due to CDK2 inhibition was tested through a comparative study with the structurally related compounds NU6230 and NU6102 (Fig. 1A). NU6230 (CDK2 IC50 = 18 μM) is an equipotent CDK to inhibitor to NU2058 (CDK2 IC50 = 17 μM), whilst NU6102 (CDK2 IC50 = 5 nM) is >1000-fold [19] more potent as an inhibitor of this enzyme. The effects of NU2058, NU6230, and NU6102 on cisplatin cytotoxicity were evaluated by treating SQ20b cells for 2 h with each compound alone at a concentration of 100 μM, followed by a further 2 h with each compound in the presence of cisplatin. All compounds were non-toxic when used alone in the absence of cisplatin, using the schedule described. The previously reported pronounced potentiation of cisplatin cytotoxicity by NU2058 in SQ20b cells [17] was confirmed (Fig. 1B). In these experiments the LC50 values (concentration of cytotoxic drug that reduces plating efficiency by 50%) were determined. NU2058 enhanced cisplatin-induced cell death with a dose modification factor (DMF) of 3.1-fold (p < 0.001, two-way ANOVA). DMF is defined as the ratio of the LC50 for cytotoxic drug alone to the LC50 for combined treatment with cytotoxic drug and NU2058 (or NU6102/NU6230).

NU6102 significantly enhanced the cytotoxicity of cisplatin (Fig. 1B), with a DMF of 2.3 (p < 0.05, two-way ANOVA). In contrast, NU6230 caused no significant enhancement of cisplatin mediated cell death (Fig. 1B, p < 0.05, two-way ANOVA). To evaluate whether the failure of NU6230 to enhance the cytotoxicity of cisplatin was due to inadequate intracellular accumulation of the compound, and hence failure to inhibit CDK2, the effects of the compounds on the phosphorylation of Rb at threonine 821 were measured, a well-characterised CDK2 phosphorylation site [24]. Attempts to re-probe the membranes for total Rb failed to give reproducible results, and so actin was used as a loading control. Cells incubated with NU6102 for 4 and 24 h showed a clear reduction in levels of phospho-Rb (Fig. 1C). NU2058 and NU6230 caused a slight decrease in Rb phosphorylation after 4 h and a clear decrease after 24 h. Thus NU6230 was at least as effective as NU2058 at decreasing the phosphorylation of Rb in a cell-based assay, and at inhibiting CDK2 in a cell-free enzyme assay, but did not potentiate cisplatin cytotoxicity. Conversely, NU6102 was markedly more effective than NU2058 in both cell-free and cell-based CDK2 assays, but was no more effective than NU2058 at potentiating cisplatin cytotoxicity. Together, these data do not support the hypothesis that the potentiation of cisplatin cytotoxicity by NU2058 is due to CDK2 inhibition.

3.2. NU2058 increased cellular levels of cisplatin and Pt–DNA adducts without affecting DNA repair

To investigate whether the increased cytotoxicity of cisplatin in the presence of NU2058 was due to effects on drug–DNA interaction, cells were harvested immediately after drug treatment, DNA extracted, and the amount of platinum bound to DNA measured using ICP-MS. Platinum–DNA adducts were linearly counted using a ColCount™ image analyser (Oxford Optronix, UK). Percentage survival was calculated relative to the survival of control cells, treated with the compound vehicle alone (DMSO).
related to extracellular cisplatin concentration, and NU2058 increased the number of platinum–DNA adducts by twofold (Fig. 2A, \( p < 0.001 \)). The formation and loss of DNA adducts over time were examined in cells treated with NU2058 or vehicle (0.1% (v/v) DMSO) for 2 h followed by a further 2 h in combination with 25 \( \mu \)M cisplatin. Cells were then washed with, and further incubated in, drug-free medium and harvested at the indicated times. As shown in Fig. 2B, NU2058 again increased the formation of platinum–DNA adducts, but did not alter their subsequent rate of removal.

To investigate whether the increase in cisplatin–DNA adduct formation induced by NU2058 was due to increased intracellular cisplatin levels, the total concentration of platinum in cells after treatment with cisplatin in the presence or absence of NU2058 was evaluated. Total cellular platinum was also linearly related to extracellular cisplatin concentration, and for any given concentration of cisplatin, NU2058 caused a 1.5-fold increase in the levels of cellular platinum (Fig. 2C, \( p < 0.001 \)). To examine whether the increased cytotoxicity of cisplatin in the presence of NU2058 was due solely to increased cisplatin–DNA adduct formation, the relationship between cisplatin–DNA adduct levels and survival was studied by combining data from Figs. 1B and 2A to evaluate the cytotoxicity as a function of DNA adduct levels. Fig. 2D shows that for equal levels of cisplatin–DNA adducts, cell survival was lower in the presence of NU2058.

### 3.3. Potentiation of cisplatin cytotoxicity by NU2058 was highly schedule dependent

To determine whether the sequence of drug administration was important for the enhancement of cisplatin cytotoxicity by NU2058, various treatment schedules were studied. Exposure to cisplatin for all schedules was for 2 h, and the sequence of cisplatin and NU2058 treatments was as described in the figure legend (Fig. 3). Schedules that did not involve overlapping of NU2058 and cisplatin exposure produced no significant potentiation of cisplatin cytotoxicity (Fig. 3A and D), and the enhancement of cisplatin cytotoxicity by NU2058 was therefore dependent on both drugs being present simultaneously (Fig. 3B and C).

### 3.4. NU2058 potentiated melphalan and monohydroxymelphalan but not temozolomide or ionising radiation

The ability of NU2058 to potentiate other DNA damaging agents was investigated. NU2058 potentiated the cytotoxic effects of melphalan (DMF of 2.2 \( p < 0.05, \) two-way ANOVA) and monohydroxymelphalan (DMF of 2.1 \( p < 0.05, \) two-way ANOVA), but not temozolomide \( p = 0.22, \) two-way ANOVA or ionising radiation \( p = 0.99, \) two-way ANOVA) (Fig. 4A–D). Since NU2058 increased cisplatin–DNA adduct levels (Fig. 2A), the effect of NU2058 on melphalan–DNA adduct levels was studied using an immunoassay based on an antibody that is specific for melphalan–guanine adducts. Although, in contrast to cisplatin, NU2058 did not affect melphalan–DNA adduct levels \( p > 0.05, \) two-way ANOVA, Fig. 5A), NU2058 did increase the cytotoxicity of the adducts formed (Fig. 5B).

### 4. Discussion

The primary aim of this study was to investigate the mechanism by which NU2058 potentiates cisplatin cytotoxicity. NU2058 was developed as an inhibitor of CDK2, and to determine whether its effect on cisplatin cytotoxicity was mediated via CDK2 inhibition, two structurally related compounds, NU6102 and NU6230, were also studied. NU6230 showed no potentiation of cisplatin (Fig. 1B) despite being similar to NU2058 in its ability to inhibit purified CDK2 (NU2058 CDK2 \( IC_{50} = 17 \) \( \mu \)M; NU6230 CDK2 \( IC_{50} = 18 \) \( \mu \)M). The lack of potentiation of cisplatin cytotoxicity was not due to poor access of NU6230 to CDK2 in cells, because NU6230 caused similar inhibition of Rb phosphorylation to NU2058, a widely used marker of CDK2 activity (Fig. 1C). In contrast to NU6230 and NU2058, NU6102 was a much more potent inhibitor of purified CDK2 (NU6102 CDK2 \( IC_{50} = 5 \) nM) and, consistent with the greater potency of the compound, exerted a much greater effect on Rb phosphorylation in SQ20b cells than did NU2058 (Fig. 1C). However, despite its greater potency as a CDK2 inhibitor, NU6102 was no more effective than NU2058 at potentiating cisplatin cytotoxicity. These data demonstrate that inhibition of CDK2 was not the mechanism by which NU2058 potentiated cisplatin cytotoxicity. The effects reported here are not specific to...
SQ20b cells because comparable potentiation of cisplatin has been observed in other human cell lines representing other types of cancer (for example LoVo and SW620, data not shown).

Since NU2058 and NU6102 inhibit CDK2 by interaction at the ATP-binding site [16,19], and ATP-binding sites are found on multiple proteins, we propose that the potentiation of cisplatin and certain other cytotoxic drugs by NU2058, was due to the interaction with another, as yet unknown ATP-binding protein, possibly a protein kinase. The lack of efficacy of NU6230 is presumably due to the large substituent at the C-8 position (Fig. 1A), and NU6230 represents an important negative control compound for further mechanistic studies. Since NU2058 is an analogue of guanine it could, in principle, become converted to a nucleotide and even incorporated into DNA. However, the large substituent at the O6-position is likely to compromise the ability of NU2058 to act as a substrate for hypoxanthine/guanine phosphoribosyl transferase, and subsequently nucleotide kinases and DNA polymerase. Nevertheless, we cannot exclude the possibility that the biological effects of NU2058 are related to its activation via the purine salvage pathway.

NU2058 also potentiated the cytotoxicity of carboplatin, oxaliplatin (data not shown) and the nitrogen mustard, melphalan. However, it did not have any effect on the sensitivity of cells to ionising radiation or temozolomide. NU2058 also potentiated the monofunctional derivative of melphalan, monohydroxymelphalan; however, as expected from previous studies with ML-1 cells [25] monohydroxymelphalan was approximately 20-fold less toxic than melphalan towards SQ20b cells (Fig. 4F). The cytotoxicity of monohydroxymelphalan, and the associated potentiation by NU2058, could not be attributed to low levels of intact bifunctional melphalan being present because HPLC analysis of the monohydroxymelphalan used showed that any such contamination was <0.2% (H. Thomas, personal communication), a level too low to account for the observed cytotoxicity. Therefore, the potentiation of monohydroxymelphalan by NU2058 demonstrates that the potentiation of melphalan by NU2058 was not solely dependent on the bifunctional properties of the latter molecule.

The lack of potentiation by NU2058 of both temozolomide (a DNA methylating agent) and ionising radiation (which involves DNA single and double strand breaks) demonstrates that the type of DNA damage is an important determinant of the ability of NU2058 to enhance cytotoxicity. Since cisplatin–DNA adduct levels were based on total platinum bound to DNA, they reflect mainly the levels of intra-strand 1,2 cross-links as these constitute the majority of adducts present on DNA [26]. DNA inter-strand cross-links could be particularly important for cytotoxicity but [27], since these only constitute about 1% of the total adducts [28], the present data cannot rule out an effect of NU2058 on repair of these specific lesions despite the lack of any effects of NU2058 on overall cisplatin adduct removal (Fig. 2B). However, as discussed above, the potentiation observed with monohydroxymelphalan unequivocally demonstrates that the effect of NU2058 does not involve a mechanism specific for DNA cross-links.

Total cellular platinum content following cisplatin exposure was increased 1.5-fold by NU2058 and this could be responsible,
fully or in part, for the increase in platinum–DNA adduct formation induced by NU2058 (Fig. 2C). NU2058 could affect cellular platinum levels either by causing an increase in cisplatin influx or a decrease in efflux, effects which in turn could cause an increase in drug–DNA interactions. The transport of cisplatin has recently been shown to be related to the transport of copper[3,4], and three copper transporters have been identified which can transport cisplatin; Ctr1, the copper influx carrier protein which passively transports copper across the cell membrane, and ATP7A and ATP7B, two ATP-dependent copper efflux transporters. However, when cell survival was related to level of platinum–DNA adducts it was shown that for equivalent adduct levels, cells treated with NU2058 showed an approximately twofold increase in cytotoxicity in relation to cells treated with cisplatin alone. This latter result suggests that there are two mechanisms involved in the potentiation of cisplatin cytotoxicity by NU2058: (1) that NU2058 affects the transport of cisplatin, resulting in increased DNA adduct formation and (2) that NU2058 alters the cellular response to the drug–DNA adducts, such that adducts are more toxic.

In contrast to the effect on cisplatin–DNA adducts, NU2058 had no effect on melphalan–DNA adduct levels (Fig. 5), and potentiation was therefore solely due to the increased cytotoxicity of the adducts that were formed. We hypothesise that the mechanism of potentiation of melphalan is the same as the second mechanism proposed above for the potentiation of cisplatin; namely, that NU2058 alters the cellular response to DNA adducts. To investigate whether the altered cellular response induced by NU2058 was related to effects on DNA repair processes, cisplatin–DNA adduct formation and loss were studied. NU2058 did not alter the rate of DNA adduct loss after removal of cisplatin. Furthermore, consistent with the lack of involvement of DNA repair in the effects of NU2058, it is particularly noteworthy that for potentiation of cisplatin by NU2058 it was necessary for both drugs be present simultaneously (Fig. 3), exposure to NU2058 directly after cisplatin, when repair would be expected to be active, having no effect. Further experiments are required to verify this hypothesis.

As shown here, the potentiation of cisplatin was independent of CDK2 inhibition, and hence it seems likely that potentiation by NU2058 was due to effects involving other protein kinases or ATP-binding proteins. Although part of the cisplatin potentiation observed was due to an effect on cisplatin transport, the major effect appears to be an alteration to the way that cells respond to DNA damage, a response that is common to cisplatin and melphalan. The lack of an effect on temozolomide and IR cytotoxicity could reflect the different cellular response mechanisms to DNA damage caused by these latter two treatments, and since the available data are not consistent with an effect on DNA damage repair, it seems likely that NU2058 is affecting early cellular responses to DNA damage. Phosphorylation events on chromatin have been shown to occur rapidly after cisplatin treatment [29], and could have a role to play in the cellular...

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**Fig. 4.** Effect of NU2058 on the cytotoxicity of various DNA damaging agents. SQ20b cells were treated with 100 μM NU2058 or vehicle (0.1% (v/v) DMSO) for 2 h and for a further 2 h in combination with either (A) melphalan, (B) monohydroxymelphalan, (C) ionising radiation or (D) temozolomide. Cells were assessed as colony forming ability 12 days after treatment. Each data point represents the mean and bars represent the standard deviation of three separate experiments.

**Fig. 5.** The effect of NU2058 on melphalan–DNA adduct formation. (A) Cells were treated with 100 μM NU2058 for 2 h followed by a further 2 h in combination with melphalan. Melphalan–DNA adduct levels were measured by immunoassay as described in Section 2. (B) Survival of cells in relation to DNA–melphalan adduct levels assessed by combining data from (A) and Fig. 4A.
response to cisplatin. An alternative explanation is that NU2058 is altering the nature or distribution of the DNA damage across the genome, although not necessarily the overall levels. Ongoing in vitro and in vivo experiments are aimed at identifying kinases that could be the targets by which NU2058 and related compounds potentiate the toxicity of cytotoxic drugs, and the therapeutic potential of cisplatin–NU2058 combinations.

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