



HAL
open science

The kinase inhibitor cyclohexylmethylguanidine (NU2058) potentiates the cytotoxicity of cisplatin by mechanisms that are independent of its effect upon CDK2

Luke R.E. Harrison, Christopher J. Ottley, D. Graham Pearson, Roger J. Griffin, Stephen R. Wedge, M. Eileen Dolan, David R. Newell, Michael J. Tilby

► To cite this version:

Luke R.E. Harrison, Christopher J. Ottley, D. Graham Pearson, Roger J. Griffin, Stephen R. Wedge, et al.. The kinase inhibitor cyclohexylmethylguanidine (NU2058) potentiates the cytotoxicity of cisplatin by mechanisms that are independent of its effect upon CDK2. *Biochemical Pharmacology*, 2009, 77 (10), pp.1586. 10.1016/j.bcp.2009.02.018 . hal-00493497

HAL Id: hal-00493497

<https://hal.science/hal-00493497>

Submitted on 19 Jun 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: The kinase inhibitor *O*⁶-cyclohexylmethylguanine (NU2058) potentiates the cytotoxicity of cisplatin by mechanisms that are independent of its effect upon CDK2

Authors: Luke R.E. Harrison, Christopher J. Ottley, D. Graham Pearson, Roger J. Griffin, Stephen R. Wedge, M. Eileen Dolan, David R. Newell, Michael J. Tilby

PII: S0006-2952(09)00119-1
DOI: doi:10.1016/j.bcp.2009.02.018
Reference: BCP 10098

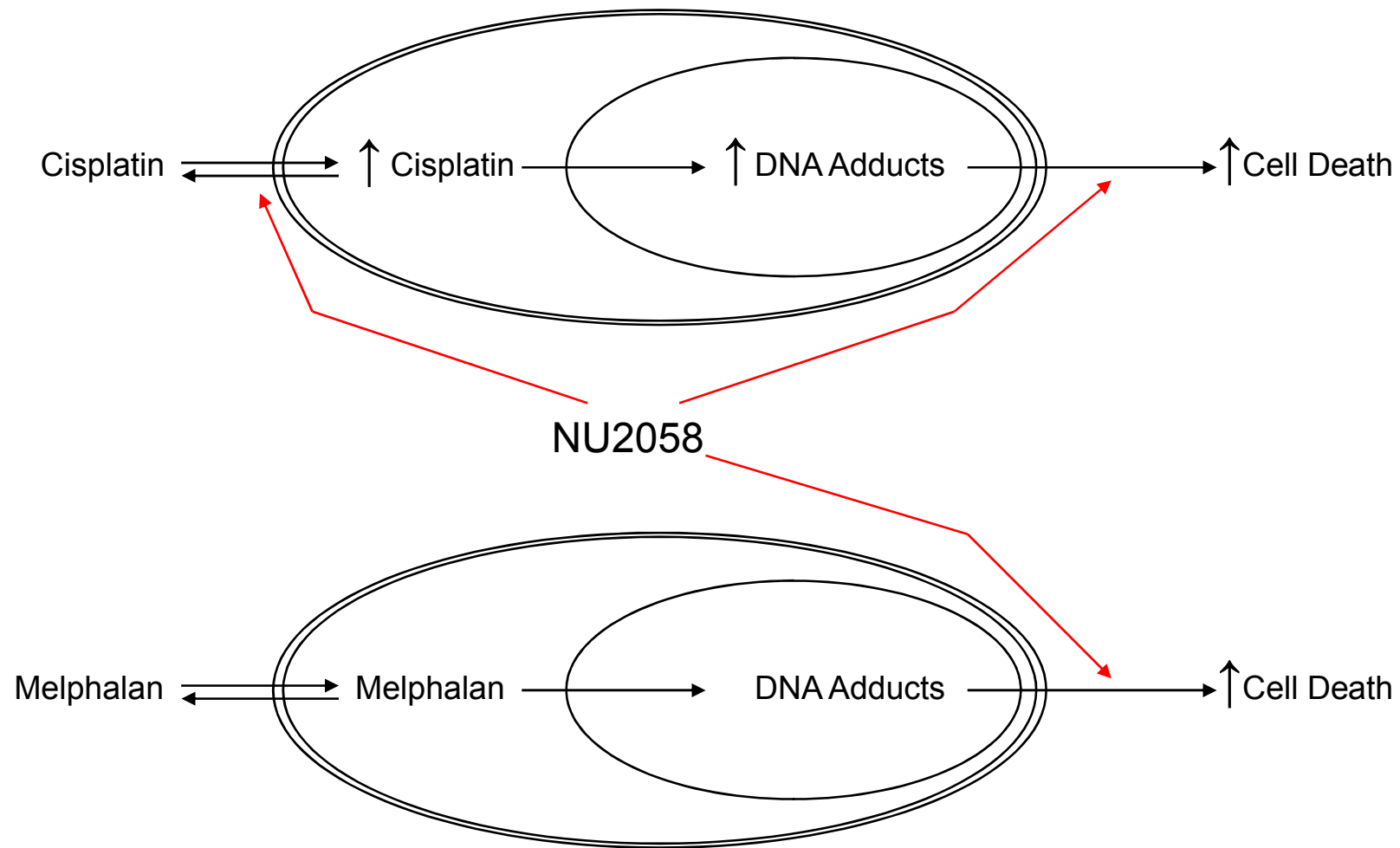
To appear in: *BCP*

Received date: 1-1-2009
Revised date: 19-2-2009
Accepted date: 19-2-2009

Please cite this article as: Harrison LRE, Ottley CJ, Pearson DG, Griffin RJ, Wedge SR, Dolan ME, Newell DR, Tilby MJ, The kinase inhibitor *O*⁶-cyclohexylmethylguanine (NU2058) potentiates the cytotoxicity of cisplatin by mechanisms that are independent of its effect upon CDK2, *Biochemical Pharmacology* (2008), doi:10.1016/j.bcp.2009.02.018

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.





1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

**The kinase inhibitor *O*⁶-cyclohexylmethylguanidine (NU2058) potentiates the
cytotoxicity of cisplatin by mechanisms that are independent of its effect upon
CDK2**

Luke R.E. Harrison, Christopher J. Ottley, D. Graham Pearson, Roger J. Griffin,

Stephen R. Wedge, M. Eileen Dolan, David R. Newell, Michael J. Tilby

Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne,

United Kingdom (L.R.E.H., R.J.G., D.R.N., M.J.T.); Department of Geological

Sciences, Durham University, Durham, United Kingdom (C.J.O., D.G.P.); Cancer

Bioscience, AstraZeneca, Alderley Park, Macclesfield, UK (S.R.W.); Department of

Medicine, Committee on Cancer Biology and Cancer Research Center, University of

Chicago, Chicago, Illinois, USA (M.E.D.).

Running title – Protein kinase inhibitors and DNA damaging agents.

Key words – cisplatin, melphalan, potentiation, kinase inhibitor, NU2058

Abbreviations used: CDK, cyclin dependent kinase; DMSO, dimethylsulphoxide;

DMF, Dose Modification Factor; HPLC, high performance liquid chromatography;

ICP-MS, inductively coupled plasma mass spectroscopy; IR, ionising radiation; Rb,

retinoblastoma protein.

Abstract

1
2 *O*⁶-Cyclohexylmethylguanine (NU2058) was developed as an inhibitor of CDK2 and
3
4 was previously shown to potentiate cisplatin cytotoxicity *in vitro*. The aim of this
5
6 study was to investigate the mechanism of cisplatin potentiation by NU2058.
7
8

9
10
11 SQ20b, head and neck cancer cells were treated for 2 hours with NU2058 (100 μ M)
12
13 and then for a further 2 hours with cisplatin and NU2058. NU2058 increased cisplatin
14
15 cytotoxicity, by clonogenic assay, with a dose modification factor (DMF) of 3.1.
16
17

18
19
20
21 NU2058 increased total intracellular platinum levels 1.5-fold, and platinum-DNA
22
23 adduct levels 2-fold. Furthermore, the cisplatin-DNA adducts formed were more toxic
24
25 in the presence of NU2058. To investigate whether the effects of NU2058 on cisplatin
26
27 adduct levels and toxicity were dependent on CDK2 activity, additional CDK2
28
29 inhibitors were tested. NU6230 (CDK2 IC₅₀ 18 μ M) was equipotent to NU2058
30
31 (CDK2 IC₅₀ 17 μ M) as a CDK2 inhibitor in cell-free and cell-based assays, yet did
32
33 not potentiate cisplatin cytotoxicity. Furthermore, NU6102 was >1000-fold more
34
35 potent than NU2058 as a CDK2 inhibitor (CDK2 IC₅₀ 5 nM) yet was no more active
36
37 than NU2058 in potentiating cisplatin.
38
39
40
41
42
43
44

45
46 NU2058 also potentiated melphalan (DMF 2.3), and monohydroxymelphalan (1.7),
47
48 but not temozolomide or ionising radiation. While NU2058 increased melphalan
49
50 cytotoxicity, it did not increase melphalan-DNA adduct formation.
51
52
53
54
55
56
57
58
59
60
61
62

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

These studies demonstrate that NU2058 alters the transport of cisplatin, causing more Pt-DNA adducts, as well as sensitizing cells to cisplatin- and melphalan-induced DNA damage. However, the effects of NU2058 are independent of CDK2 inhibition.

Accepted Manuscript

1. Introduction

1
2 Platinum compounds such as cisplatin and carboplatin remain amongst the most
3
4 widely used chemotherapeutic drugs in the treatment many tumours types, including
5
6 head and neck, testicular, lung, and ovarian carcinomas. For example, the
7
8 combination of bleomycin, etoposide and cisplatin is considered curative therapy for
9
10 testicular cancer, with survival rates of over 95% [1]. Like most cytotoxic anticancer
11
12 drugs, cisplatin acts *via* the induction of DNA damage involving a number of types of
13
14 DNA adducts [2].
15
16
17
18
19
20
21

22 Although cisplatin has clinical utility against some tumour types it has little activity
23
24 against others. Furthermore, initially sensitive tumours can become resistant to
25
26 platinum complexes. Acquired as well as intrinsic cisplatin resistance can be caused by
27
28 several mechanisms, including altered drug uptake, increased activity of cellular
29
30 detoxification systems and DNA repair [3-8]. Modulation of cellular responses to
31
32 established drugs such as cisplatin have the potential to both improve efficacy and
33
34 attenuate toxicity.
35
36
37
38
39
40

41 Protein kinases are involved in a wide range of cellular events including cell growth,
42
43 DNA repair, replication and cell survival. Many protein kinases are deregulated in
44
45 the development and progression of cancer, and protein kinase inhibitors are currently
46
47 being used therapeutically to exploit the molecular pathology of neoplasms.
48
49
50

51 Trastuzumab and imatinib are two clinically successful anti-cancer drugs which act
52
53 *via* inhibition of kinase signalling pathways [9, 10]. Encouraged by the success of
54
55 imatinib and trastuzumab, the identification of protein kinases that are deregulated in
56
57
58
59
60
61
62

1 cancer and the development of kinase inhibitors is currently a major drug discovery
2 priority.
3

4
5
6
7 Cyclin dependent kinases (CDK), and their partner cyclins, are key proteins in the
8 regulation of the cell cycle and are frequently deregulated in cancer cells [11]. The
9 CDKs have been investigated as targets for cancer therapy and the antitumour activity
10 of CDK inhibitors has been demonstrated in preclinical models, both *in vitro* and *in*
11 *vivo* [12]. However, CDK inhibitors have so far shown little or no single agent
12 clinical activity, with the possible exception of flavopiridol for the treatment of
13 chronic lymphocytic leukaemia [13]. These latter data are consistent with preclinical
14 studies, where inhibition of CDKs by flavopiridol was shown to induce cell death
15 [14]. It has also been suggested that CDK inhibitors could be used to increase the
16 efficacy of cytotoxic therapies by modulating cellular responses, possibly by
17 recruiting or holding cells in a drug sensitive phase of the cell cycle [15].
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35

36 *O*⁶-Cyclohexylmethylguanine (NU2058, Fig. 1A) is a compound that was identified in
37 a programme to develop potent and selective inhibitors of CDK2. NU2058 showed
38 modest inhibition of CDK2 with an IC₅₀ (concentration required to give 50%
39 inhibition of CDK2 in an isolated enzyme assay) value of 17 μM [16]. NU2058 has
40 previously been shown to potentiate the cytotoxicity of cisplatin *in vitro* [17] and it
41 was hypothesised that inhibition of CDK2 was responsible for this effect.
42
43
44
45
46
47
48
49
50
51
52

53 In the present work, we tested the hypothesis that CDK2 inhibition was responsible
54 for the potentiation of cisplatin cytotoxicity by NU2058 through the use of two
55 structurally-related compounds; NU6102 and NU6230 (Fig. 1A). Furthermore, to
56
57
58
59
60
61
62

1 improve our understanding of the mechanism of potentiation of cisplatin by NU2058
2 we also investigated: 1) whether NU2058 affected cisplatin-DNA interactions and
3 cellular drug uptake; 2) if NU2058 potentiated the cytotoxicity of other DNA
4 damaging agents; 3) whether the effect of NU2058 on potentiation of cisplatin was
5 schedule dependent.
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 per min.). NU2058, NU6230, NU6102 were synthesised as described previously [16, 19] and stored as 100 mM stock solutions in DMSO at -20°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 µg/ml hydrocortisone, as previously described [20]. Cells were grown in the presence of penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37 °C and 5% CO₂.

2.3 Colony formation assay

Cells were seeded into 6 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 µM of either NU2058, NU6102, NU6230 or DMSO (0.1% (v/v) final concentration) for 2 hours followed by a further 2 hours in the additional presence of cytotoxic drugs, unless otherwise indicated. For the radiation experiments, cells were treated for a total of 4 hours with NU2058, and irradiated after the first 2 hours. After treatment, the cells were washed twice with PBS, trypsinised, and replated into 100

1 mm Petri dishes at various cell densities (300 – 50,000 cells per plate). After
2 approximately 12 days, media was removed, and cells were fixed with Carnoy's
3 reagent (75% (v/v) methanol, 25% (v/v) acetic acid), stained with crystal violet (0.4%
4 (w/v) in water) and colonies were counted using a ColCount™ image analyser
5 (Oxford Optronix, UK). Percentage survival was calculated relative to the survival of
6 control cells, treated with the compound vehicle alone (DMSO).
7
8
9
10
11
12
13
14
15
16

17 **2.4 Western blotting**

18 Phosphorylation of retinoblastoma protein (Rb) was measured in cells after incubation
19 with each compound or DMSO. Exponentially growing cells were treated in 15 cm
20 dishes with 100 µM of either NU2058, NU6230, NU6102 or vehicle (0.1 % (v/v)
21 DMSO) for a period of 4 or 24 hours. After treatment, cells were washed twice with
22 ice cold PBS, and lysed with Laemmli buffer [21]. Samples were sonicated and
23 protein concentration quantified using BCA protein assay (Pierce, USA). Equal
24 amounts of protein (15 µg) were separated on a 4-20% (w/v) tris-glycine SDS
25 polyacrylamide gel (Invitrogen, UK) and transferred to a PVDF blotting membrane
26 (BioRad, USA). The membrane was blocked in 5% (w/v) milk in tris buffered saline
27 containing 0.2% (v/v) tween (TBST), and the primary monoclonal phosphospecific
28 (pRb-T⁸²¹) antibody (Biosource, CA, Cat. No. 44-582G) was added at a dilution factor
29 of 1:1000 for two hours, washed with TBST, and incubated with a rabbit secondary
30 antibody (DakoCytomation, Denmark, Cat. No. P0217) in TBST containing 5% (w/v)
31 milk for a further two hours. The ECL system (Pierce, USA) was used for detection of
32 phospho-Rb. The image was captured with photographic film and developed. For
33 measurement of actin, membranes were incubated as above but with a primary anti-
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 actin (Sigma, UK. Cat. No. A4700) and with a rabbit anti-mouse secondary antibody
2 (DakoCytomation, Denmark, Cat. No. P0260).
3
4
5
6

7 **2.5 Measurement of total cellular platinum and platinum-DNA adducts**

8
9 Actively growing cells were treated with NU2058 (100 μ M) or DMSO control for 2
10 hours and then a further 2 hours in the additional presence of cisplatin (0-50 μ M).
11
12

13 After treatment, cells were either washed twice with ice-cold PBS, trypsinised and
14 collected by centrifugation, or washed twice with warm growth medium and further
15 incubated at 37°C for periods up to 24 hours. For platinum-DNA adduct analysis,
16 DNA was extracted from cells using the Qiagen DNeasy kit according to the
17 manufacturer's instructions (Qiagen, UK). Isolated DNA was quantified by measuring
18 OD₂₆₀ using a ND-1000 Spectrophotometer (NanoDrop, USA). Extracted DNA was
19 hydrolysed with 3.5% nitric acid (v/v) Spectrosol grade (Merck, UK) overnight at
20 70°C, prior to analysis by inductively coupled plasma mass spectroscopy (ICP-MS).
21 For measurement of total cellular platinum, cell pellets were sonicated and protein
22 measured as described above. Samples were then incubated with 1 M NaOH for 1
23 hour at 60°C, followed by an overnight acid hydrolysis with 1 M nitric acid at 70°C.
24 Platinum concentrations in samples were determined by ICP-MS as described
25 previously [22], and compared to a standard curve analysed on the same day to
26 correct for any instrument drift.
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50

51 **2.6 Measurement of melphalan-DNA adducts**

52 For measurement of melphalan-DNA adducts, cells were pre-treated with 100 μ M
53 NU2058 or vehicle (0.1% (v/v) DMSO) for two hours followed by a further two hours
54 in the additional presence of melphalan (0 - 50 μ M). After drug treatment, cells were
55
56
57
58
59
60
61
62
63
64
65

1 washed twice with ice-cold PBS and released using trypsin. Cells were then
2 centrifuged and washed once more with PBS, and pellets were stored at -20°C until
3 required. DNA was extracted using the phenol hydroxyapatite method as described by
4 Tilby *et al* [18]. DNA concentration was determined by measuring OD₂₆₀ using a ND-
5 1000 Spectrophotometer (NanoDrop, USA). Melphalan-DNA adducts were quantified
6 using a competitive ELISA as described previously [23]. Briefly, several dilutions of
7 DNA from treated cells were mixed with a constant amount of MP5/73 antibody,
8 which was specific for melphalan-guanine adducts on DNA, and incubated in wells
9 coated with a standard amount of melphalan-treated DNA. The amount of antibody
10 that bound to the wells was determined by a fluorogenic enzyme assay. The quantities
11 of adducts per assay well that caused a 50% reduction of the assay signal were
12 determined by a curve fitting procedure.
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 CDK2 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 hours with each compound alone at a concentration of 100 μM, followed by a further 2 hours 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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 hours showed a clear reduction in levels of phospho-Rb (Fig. 1C). NU2058 and NU6230 caused a slight decrease in Rb phosphorylation after four hours and a clear decrease after 24 hours. 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 related to extracellular cisplatin concentration, and NU2058 increased the number of platinum-DNA adducts by 2-fold (Fig. 2A, $p < 0.001$). The formation and loss of DNA-adducts over time was examined in cells treated with NU2058 or vehicle (0.1% (v/v) DMSO) for 2 hours followed by a further 2 hours in combination with 25 μ M cisplatin. Cells were then washed with, and further incubated in, drug-free medium and harvested at

1 the indicated times. As shown in Figure 2B, NU2058 again increased the formation
2 of platinum-DNA adducts, but did not alter their subsequent rate of removal.
3
4

5
6
7 To investigate whether the increase in cisplatin-DNA adduct formation induced by
8
9
10 NU2058 was due to increased intracellular cisplatin levels, the total concentration of
11
12 platinum in cells after treatment with cisplatin in the presence or absence of NU2058
13
14 was evaluated. Total cellular platinum was also linearly related to extracellular
15
16 cisplatin concentration, and for any given concentration of cisplatin, NU2058 caused
17
18 a 1.5 fold increase in the levels of cellular platinum (Fig. 2C, $p < 0.001$). To examine
19
20 whether the increased cytotoxicity of cisplatin in the presence of NU2058 was due
21
22 solely to increased cisplatin-DNA adduct formation, the relationship between
23
24 cisplatin-DNA adduct levels and survival was studied by combining data from
25
26
27
28 Figures 1B and 2A to evaluate the cytotoxicity as a function of DNA adduct levels.
29
30
31 Figure 2D shows that for equal levels of cisplatin-DNA adducts, cell survival was
32
33
34 lower in the presence of NU2058.
35
36
37
38

39 **3.3 Potentiation of cisplatin cytotoxicity by NU2058 was highly schedule** 40 41 **dependent**

42
43 To determine whether the sequence of drug administration was important for the
44
45 enhancement of cisplatin cytotoxicity by NU2058, various treatment schedules were
46
47 studied. Exposure to cisplatin for all schedules was for two hours, and the sequence of
48
49 cisplatin and NU2058 treatments was as described in the figure legend (Fig. 3).
50
51
52 Schedules that involved no overlap of NU2058 and cisplatin exposure produced no
53
54
55 significant potentiation of cisplatin cytotoxicity (Fig. 3A and 3D), and the
56
57
58
59
60
61
62
63
64
65

1 enhancement of cisplatin cytotoxicity by NU2058 was therefore dependent on both
2 drugs being present simultaneously (Fig. 3B and C).
3
4
5
6

7 **3.4 NU2058 potentiated melphalan and monohydroxymelphalan but not** 8 **temozolomide or ionising radiation** 9

10 The ability of NU2058 to potentiate other DNA damaging agents was investigated.
11
12 NU2058 potentiated the cytotoxic effects of melphalan (DMF of 2.2 ($p < 0.05$, two-
13 way ANOVA)) and monohydroxymelphalan (DMF of 2.1 ($p < 0.05$, two-way
14 ANOVA)), but not temozolomide ($p = 0.22$, two-way ANOVA) or ionising radiation
15
16 ($p = 0.99$, two-way ANOVA) (Fig. 4A-D). Since NU2058 increased cisplatin-DNA
17
18 adduct levels (Fig. 2A), the effect of NU2058 on melphalan-DNA adduct levels was
19
20 studied using an immunoassay based on an antibody that is specific for melphalan-
21
22 guanine adducts. Although, in contrast to cisplatin, NU2058 did not affect
23
24 melphalan-DNA adduct levels ($p > 0.05$, two-way ANOVA, Fig. 5A), NU2058 did
25
26 increase the cytotoxicity of the adducts formed (Fig. 5B).
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

4. Discussion

1
2 The primary aim of this study was to investigate the mechanism by which NU2058
3
4 potentiates cisplatin cytotoxicity. NU2058 was developed as an inhibitor of CDK2,
5
6 and to determine whether its effect on cisplatin cytotoxicity was mediated *via* CDK2
7
8 inhibition, two structurally-related compounds, NU6102 and NU6230, were also
9
10 studied. NU6230 showed no potentiation of cisplatin (Fig. 1B) despite being similar
11
12 to NU2058 in its ability to inhibit purified CDK2 (NU2058 CDK2 IC₅₀ = 17 μM;
13
14 NU6230 CDK2 IC₅₀ = 18 μM). The lack of potentiation of cisplatin cytotoxicity was
15
16 not due to poor access of NU6230 to CDK2 in cells, because NU6230 caused similar
17
18 inhibition of Rb phosphorylation to NU2058, a widely used marker of CDK2 activity
19
20 (Fig. 1C). In contrast to NU6230 and NU2058, NU6102 was a much more potent
21
22 inhibitor of purified CDK2 (NU6102 CDK2 IC₅₀ 5 nM) and, consistent with the
23
24 greater potency of the compound, exerted a much greater effect on Rb
25
26 phosphorylation in SQ20b cells than did NU2058 (Fig. 1C). However, despite its
27
28 greater potency as a CDK2 inhibitor, NU6102 was no more effective than NU2058 at
29
30 potentiating cisplatin cytotoxicity. These data demonstrate that inhibition of CDK2
31
32 was not the mechanism by which NU2058 potentiated cisplatin cytotoxicity. The
33
34 effects reported here are not specific to SQ20b cells because comparable potentiation
35
36 of cisplatin has been observed in other human cell lines representing other types of
37
38 cancer (for example LoVo and SW620, data not shown).
39
40
41
42
43
44
45
46
47
48
49
50

51 Since NU2058 and NU6102 inhibit CDK2 by interaction at the ATP binding site [16,
52
53 19], and ATP binding sites are found on multiple proteins, we propose that the
54
55 potentiation of cisplatin and certain other cytotoxic drugs by NU2058, was due to the
56
57 interaction with another, as yet unknown ATP-binding protein, possibly a protein
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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.

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

14 Total cellular platinum content following cisplatin exposure was increased 1.5 fold by
15 NU2058 and this could be responsible, fully or in part, for the increase in platinum-
16 DNA adduct formation induced by NU2058 (Fig. 2C). NU2058 could affect cellular
17 platinum levels either by causing an increase in cisplatin influx or a decrease in
18 efflux, effects which in turn could cause an increase in drug-DNA interactions. The
19 transport of cisplatin has recently been shown to be related to the transport of copper
20 [3, 4], and three copper transporters have been identified which can transport
21 cisplatin; Ctr1, the copper influx carrier protein which passively transports copper
22 across the cell membrane, and ATP7A and ATP7B, two ATP-dependent copper
23 efflux transporters. However, when cell survival was related to level of platinum-
24 DNA adducts it was shown that for equivalent adduct levels, cells treated with

1 NU2058 showed an approximately 2-fold increase in cytotoxicity in relation to cells
2 treated with cisplatin alone. This latter result suggests that there are two mechanisms
3 involved in the potentiation of cisplatin cytotoxicity by NU2058, 1) that NU2058
4 affects the transport of cisplatin, resulting in increased DNA adduct formation, and 2)
5 that NU2058 alters the cellular response to the drug-DNA adducts, such that adducts
6 are more toxic.
7
8
9
10
11
12
13
14
15
16

17 In contrast to the effect on cisplatin-DNA adducts, NU2058 had no effect on
18 melphalan-DNA adduct levels (Fig. 5), and potentiation was therefore solely due to
19 the increased cytotoxicity of the adducts that were formed. We hypothesise that the
20 mechanism of potentiation of melphalan is the same as the second mechanism
21 proposed above for the potentiation of cisplatin; namely, that NU2058 alters the
22 cellular response to DNA adducts. To investigate whether the altered cellular response
23 induced by NU2058 was related to effects on DNA repair processes, cisplatin-DNA
24 adduct formation and loss were studied. NU2058 did not alter the rate of DNA adduct
25 loss after removal of cisplatin. Furthermore, consistent with the lack of involvement
26 of DNA repair in the effects of NU2058, it is particularly noteworthy that for
27 potentiation of cisplatin by NU2058 it was necessary for both drugs be present
28 simultaneously (Fig. 3), exposure to NU2058 directly after cisplatin, when repair
29 would be expected to be active, having no effect. Further experiments are required to
30 verify this hypothesis.
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50

51 As shown here, the potentiation of cisplatin was independent of CDK2 inhibition, and
52 hence it seems likely that potentiation by NU2058 was due to effects involving other
53 protein kinases or ATP binding proteins. Although part of the cisplatin potentiation
54 observed was due to an effect on cisplatin transport, the major effect appears to be an
55
56
57
58
59
60
61
62
63
64
65

1 alteration to the way that cells respond to DNA damage, a response that is common to
2 cisplatin and melphalan. The lack of an effect on temozolomide and IR cytotoxicity
3
4 could reflect the different cellular response mechanisms to DNA damage caused by
5
6 these latter two treatments, and since the available data are not consistent with an
7
8 effect on DNA damage repair, it seems likely that NU2058 is affecting early cellular
9
10 responses to DNA damage. Phosphorylation events on chromatin have been shown to
11
12 occur rapidly after cisplatin treatment [29], and could have a role to play in the
13
14 cellular response to cisplatin. An alternative explanation is that NU2058 is altering the
15
16 nature or distribution of the DNA damage across the genome, although not
17
18 necessarily the overall levels. Ongoing *in vitro* and *in vivo* experiments are aimed at
19
20 identifying kinases that could be the targets by which NU2058 and related compounds
21
22 potentiate the toxicity of cytotoxic drugs, and the therapeutic potential of cisplatin-
23
24 NU2058 combinations.
25
26
27
28
29
30

31 32 33 Acknowledgement

34
35
36 The authors would like to acknowledge AstraZeneca, the Medical Research Council,
37
38 and Cancer Research UK for funding this project.
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure 1

1
2 A) Structures of NU2058, NU6102, and NU6230 and CDK2 IC₅₀ B) Effect of
3
4 NU2058, NU6102 and NU6230 on the cytotoxicity of cisplatin. SQ20b cells were
5
6 pretreated with each compound alone (100 μ M) or vehicle (0.1% (v/v) DMSO) for 2
7
8 hours and for a further 2 hours in combination with cisplatin. After treatment cells
9
10 were assessed for their colony forming ability after 12 days. Points are the mean, and
11
12 bars are the standard deviation of three separate experiments. C) Effect of compounds
13
14 on phosphorylation of retinoblastoma protein. Cells were treated with each compound
15
16 (100 μ M) for four or 24 hours before analysis by Western blotting for T⁸²¹-phospho-
17
18 Rb. Results from three independent experiments are presented.
19
20
21
22
23
24
25
26

Figure 2

27
28 The effect of NU2058 on DNA-cisplatin adduct levels and cisplatin uptake. A)
29
30 Cisplatin-DNA adduct levels after treatment of SQ20b cells with 100 μ M NU2058 or
31
32 vehicle (0.1% (v/v) DMSO) for 2 hours followed by a further 2 hours in the presence
33
34 of cisplatin. Total platinum-DNA adduct levels were measured using ICP-MS. B)
35
36 Cisplatin-DNA adduct formation and loss over time. Cells were treated with 100 μ M
37
38 NU2058 or vehicle (0.1% (v/v) DMSO) between -4 to 0 hours, with cisplatin (25 μ M)
39
40 present between -2 and 0 hours. Cells were then incubated in drug free media for up
41
42 to 24 hours. C) Cellular cisplatin uptake. Cells were treated as described in A), pellets
43
44 were sonicated and total cellular platinum determined as described in methods. D)
45
46 Survival of cells in relation to DNA-cisplatin adduct levels. Values were generated
47
48 using data from Figures 1B) and 2A).
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

In Figures 2A-C points are the mean of 3 independent experiments, and bars the standard deviation. In Figures 2A and 2C the lines were fitted by linear regression analysis.

Figure 3

Effect of NU2058 treatment schedules on cisplatin cytotoxicity. SQ20b cells were treated with 100 μ M NU2058, or vehicle alone (0.1% (v/v) DMSO). Treatment with cisplatin was for two hours, and NU2058 for 4 hours, and the schedules were as follows; A) NU2058 for four hours prior to cisplatin; B) Two hour pre-treatment with NU2058 followed by two hour co-incubation with cisplatin (the schedule in Figures 1 and 2); C) A two hour co-incubation followed by a two hour post-cisplatin incubation with NU2058 alone; D) A four hour incubation with NU2058 after cisplatin.

Cytotoxicity was measured by using a colony formation assay. Each data point represents the mean and bars represent the standard deviation of three separate experiments. Solid bars represent NU2058 (100 μ M) or vehicle (0.1 % (v/v) DMSO), checked bars indicate cisplatin.

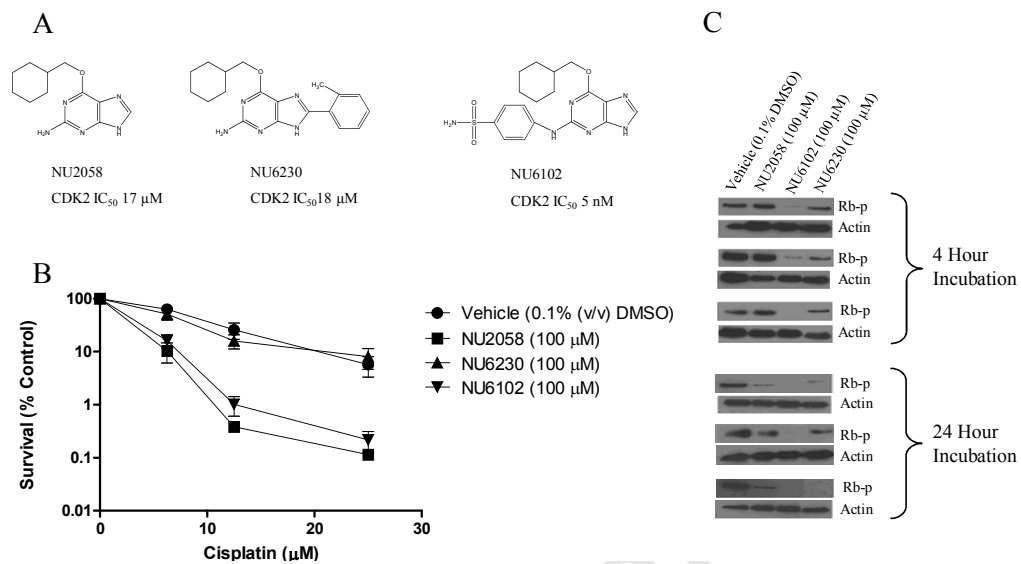
Figure 4

Effect of NU2058 on the cytotoxicity of various DNA damaging agents. SQ20b cells were treated with 100 μ M NU2058 or vehicle (0.1% (v/v) DMSO) for 2 hours and for a further 2 hours in combination with either A) melphalan; B) monohydroxymelphalan; C) ionising radiation; 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.

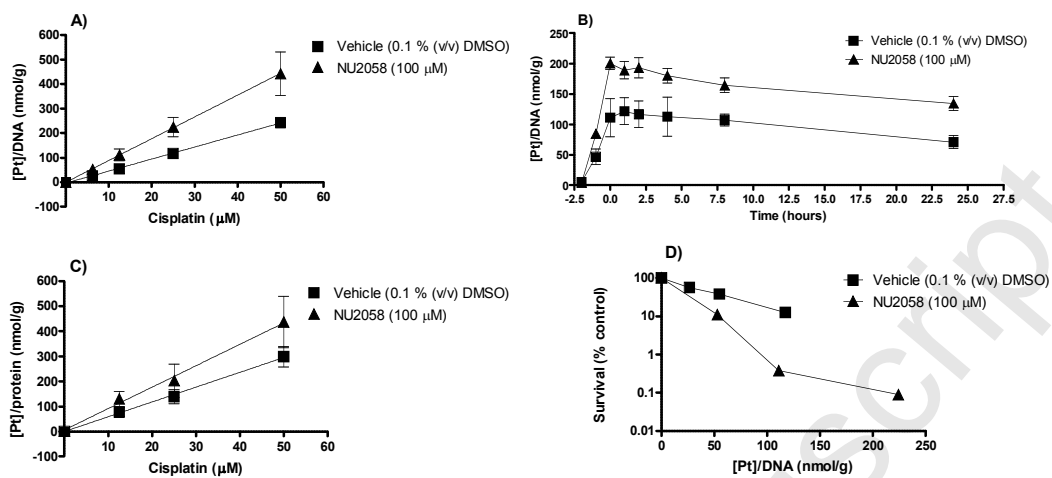
1
2
3
4
5 Figure 5
6

7 The effect of NU2058 on melphalan-DNA adduct formation A) Cells were treated
8 with 100 μ M NU2058 for 2 hours followed by a further 2 hours in combination with
9 melphalan. Melphalan-DNA adduct levels were measured by immunoassay as
10 described in the methods. B) Survival of cells in relation to DNA-melphalan adduct
11 levels assessed by combining data from figure 5A and figure 4A.
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

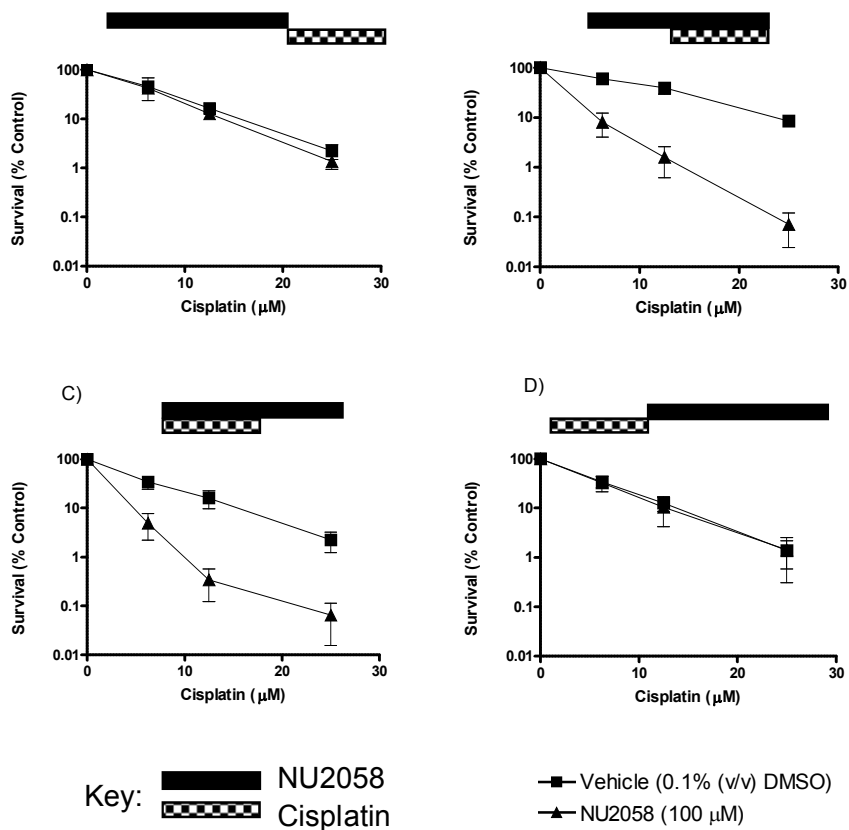
Luke Harrison. Figure 1



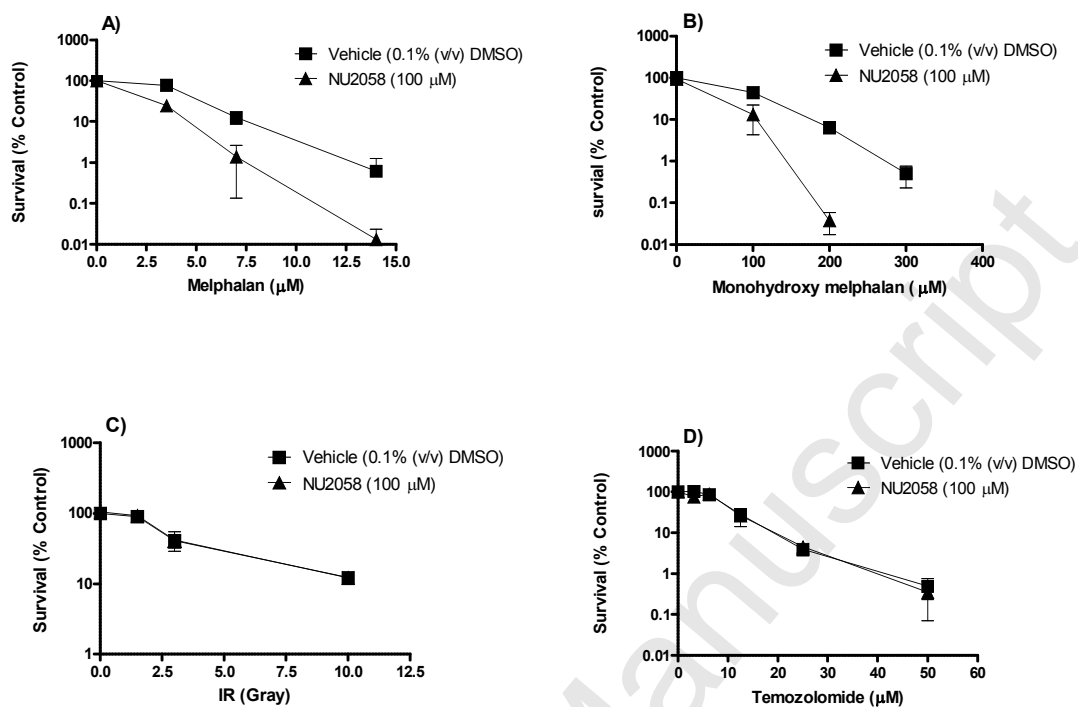
Luke Harrison. Figure 2



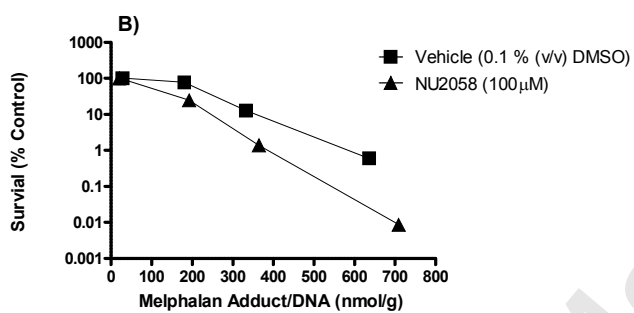
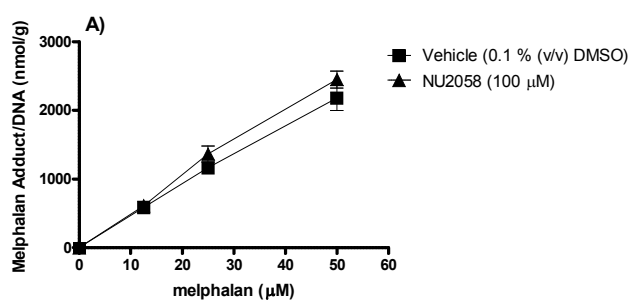
Luke Harrison. Figure 3



Luke Harrison. Figure 4



Luke Harrison. Figure 5



References

- 1
2
3 [1] Einhorn LH. Curing metastatic testicular cancer. *Proc Natl Acad Sci U S A*
4 2002;99:4592-5 [Scopus](#).
- 5
6 [2] Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. *Nat Rev*
7 *Drug Discov* 2005;4:307-20 [Scopus](#).
- 8
9 [3] Safaei R, Holzer AK, Katano K, Samimi G, Howell SB. The role of copper
10 transporters in the development of resistance to Pt drugs. *J Inorg Biochem*
11 2004;98:1607-13 [Scopus](#).
- 12
13 [4] Safaei R, Howell SB. Copper transporters regulate the cellular pharmacology and
14 sensitivity to Pt drugs. *Crit Rev Oncol Hematol* 2005;53:13-23 [Scopus](#).
- 15
16 [5] Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance.
17 *Oncogene* 2003;22:7265-79 [Scopus](#).
- 18
19 [6] Rabik CA, Dolan ME. Molecular mechanisms of resistance and toxicity
20 associated with platinating agents. *Cancer Treat Rev* 2007;33:9-23 [Scopus](#).
- 21
22 [7] Boulikas T, Vougiouka M. Cisplatin and platinum drugs at the molecular level.
23 (Review). *Oncol Rep* 2003;10:1663-82 [Scopus](#).
- 24
25 [8] Fink D, Nebel S, Aebi S, Zheng H, Cenni B, Nehme A, et al. The role of DNA
26 mismatch repair in platinum drug resistance. *Cancer Res* 1996;56:4881-6 [Scopus](#).
- 27
28 [9] Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith
29 I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer.
30 *N Engl J Med* 2005;353:1659-72 [Scopus](#).
- 31
32 [10] Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N,
33 et al. Five-year follow-up of patients receiving imatinib for chronic myeloid
34 leukemia. *N Engl J Med* 2006;355:2408-17 [Scopus](#).
- 35
36 [11] Shapiro GI. Cyclin-dependent kinase pathways as targets for cancer treatment.
37 *J Clin Oncol* 2006;24:1770-83 [Scopus](#).
- 38
39 [12] Drees M, Dengler WA, Roth T, Labonte H, Mayo J, Malspeis L, et al.
40 Flavopiridol (L86-8275): selective antitumor activity in vitro and activity in vivo
41 for prostate carcinoma cells. *Clin Cancer Res* 1997;3:273-9 [Scopus](#).
- 42
43 [13] Byrd JC, Lin TS, Dalton JT, Wu D, Phelps MA, Fischer B, et al. Flavopiridol
44 administered using a pharmacologically derived schedule is associated with
45 marked clinical efficacy in refractory, genetically high-risk chronic lymphocytic
46 leukemia. *Blood* 2007;109:399-404 [Scopus](#).
- 47
48 [14] Shapiro GI, Koestner DA, Matranga CB, Rollins BJ. Flavopiridol induces cell
49 cycle arrest and p53-independent apoptosis in non-small cell lung cancer cell
50 lines. *Clin Cancer Res* 1999;5:2925-38 [Scopus](#).
- 51
52 [15] Bible KC, Kaufmann SH. Cytotoxic synergy between flavopiridol (NSC
53 649890, L86-8275) and various antineoplastic agents: the importance of sequence
54 of administration. *Cancer Res* 1997;57:3375-80 [Scopus](#).
- 55
56 [16] Arris CE, Boyle FT, Calvert AH, Curtin NJ, Endicott JA, Garman EF, et al.
57 Identification of novel purine and pyrimidine cyclin-dependent kinase inhibitors
58
59
60
61
62
63
64
65

- with distinct molecular interactions and tumor cell growth inhibition profiles. *J Med Chem* 2000;43:2797-804 [Scopus](#).
- [17] Fishel ML, Newell DR, Griffin RJ, Davison R, Wang LZ, Curtin NJ, et al. Effect of cell cycle inhibition on Cisplatin-induced cytotoxicity. *J Pharmacol Exp Ther* 2005;312:206-13 [Scopus](#).
- [18] Tilby MJ, Newell DR, Viner C, Selby PJ, Dean CJ. Application of a sensitive immunoassay to the study of DNA adducts formed in peripheral blood mononuclear cells of patients undergoing high-dose melphalan therapy. *Eur J Cancer* 1993;29A:681-6 [Scopus](#).
- [19] Davies TG, Bentley J, Arris CE, Boyle FT, Curtin NJ, Endicott JA, et al. Structure-based design of a potent purine-based cyclin-dependent kinase inhibitor. *Nat Struct Biol* 2002;9:745-9 [Scopus](#).
- [20] Fishel ML, Delaney SM, Friesen LD, Hansen RJ, Zuhowski EG, Moschel RC, et al. Enhancement of platinum-induced cytotoxicity by O6-benzylguanine. *Mol Cancer Ther* 2003;2:633-40 [Scopus](#).
- [21] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5 [Scopus](#).
- [22] Meczes EL, Azim-Araghi A, Ottley CJ, Pearson DG, Tilby MJ. Specific adducts recognised by a monoclonal antibody against cisplatin-modified DNA. *Biochem Pharmacol* 2005;70:1717-25 [Scopus](#).
- [23] Tilby MJ, Styles JM, Dean CJ. Immunological detection of DNA damage caused by melphalan using monoclonal antibodies. *Cancer Res* 1987;47:1542-6 [Scopus](#).
- [24] Zarkowska T, Mittnacht S. Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. *J Biol Chem* 1997;272:12738-46 [Scopus](#).
- [25] Gould KA, Nixon C, Tilby MJ. p53 elevation in relation to levels and cytotoxicity of mono- and bifunctional melphalan-DNA adducts. *Mol Pharmacol* 2004;66:1301-9 [Scopus](#).
- [26] Fichtinger-Schepman AM, Lohman PH, Reedijk J. Detection and quantification of adducts formed upon interaction of diamminedichloroplatinum (II) with DNA, by anion-exchange chromatography after enzymatic degradation. *Nucleic Acids Res* 1982;10:5345-56 [Scopus](#).
- [27] Knox RJ, Friedlos F, Lydall DA, Roberts JJ. Mechanism of cytotoxicity of anticancer platinum drugs: evidence that cis-diamminedichloroplatinum(II) and cis-diammine-(1,1-cyclobutanedicarboxylato)platinum(II) differ only in the kinetics of their interaction with DNA. *Cancer Res* 1986;46:1972-9 [Scopus](#).
- [28] Roberts JJ, Friedlos F. Quantitative estimation of cisplatin-induced DNA interstrand cross-links and their repair in mammalian cells: relationship to toxicity. *Pharmacol Ther* 1987;34:215-46 [Scopus](#).
- [29] Wang D, Lippard SJ. Cisplatin-induced post-translational modification of histones H3 and H4. *J Biol Chem* 2004;279:20622-5 [Scopus](#).