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Inhibition of PARP-1 enhances the toxicity of [¹³¹I]MIBG/topotecan combination therapy to cells and xenografts which express the noradrenaline transporter

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ABSTRACT

Targeted radiotherapy using [¹³¹I]meta-iodobenzylguanidine ([¹³¹I]MIBG) has produced remissions in some neuroblastoma patients. We previously reported that combining [¹³¹I]MIBG with the topoisomerase I (**Topo-I**) inhibitor topotecan induced long-term DNA damage and supra-additive toxicity to NAT-expressing cells and xenografts. This combination treatment is undergoing clinical evaluation. This present study investigated the potential of PARP-1 inhibition, *in vitro* and *in vivo*, to further enhance [¹³¹I]MIBG/topotecan efficacy.

Methods: Combinations of topotecan and the PARP-1 inhibitor PJ34 were assessed for synergism *in vitro* by combination-index analysis in SK-N-BE(2c) (neuroblastoma) and UVW/NAT (NAT-transfected glioma) cells. Three treatment schedules were evaluated: topotecan administered (1) 24 h before, (2) 24 h after, or (3) simultaneously with PJ34. Using similar scheduling, combinations of PJ34 & [¹³¹I]MIBG and PJ34 & [¹³¹I]MIBG/topotecan were also assessed. *In vivo* efficacy was measured by growth delay of tumour xenografts. We also assessed DNA damage by γ H2AX assay, cell cycle progression by FACS analysis and PARP-1 activity in treated cells.

Results: *In vitro*, only simultaneous administration of topotecan and PJ34 or PJ34 and [¹³¹I]MIBG induced supra-additive toxicity in both cells lines. All scheduled combinations of PJ34 & [¹³¹I]MIBG/topotecan induced supra-additive toxicity and increased DNA damage in SK-N-BE(2c) cells, but

only simultaneous administration induced enhanced efficacy in UVW/NAT cells. PJ34 & [¹³¹I]MIBG/topotecan combination treatment induced G₂ arrest in all cell lines, regardless of the schedule of delivery. *In vivo*, simultaneous administration of PJ34 & [¹³¹I]MIBG/topotecan significantly delayed the growth of SK-N-BE(2c) and UVW/NAT xenografts compared to [¹³¹I]MIBG/topotecan therapy.

Conclusion: The anti-tumor efficacy of topotecan, [¹³¹I]MIBG and [¹³¹I]MIBG/topotecan combination treatment was increased by PARP-1 inhibition *in vitro* and *in vivo*.

Neuroblastoma is a heterogeneous disease, and using age, stage and other biological characteristics, patients can be assigned to various risk groups (1). There is evidence that, over time, outcomes are gradually improving (2). However, for patients with high-risk disease, outcomes remain poor (3, 4) and innovative therapies are required, especially for those whose disease fails to respond well to induction chemotherapy (5).

Approximately 90% of neuroblastoma tumour cells express the noradrenaline transporter (NAT), a 12-spanning integral membrane protein responsible for the active intracellular accumulation of catecholamine neurotransmitters. Meta-iodobenzylguanidine (MIBG), a derivative of the adrenergic neurone-blocking drugs bretylium and guanethidine, is a structural analogue of noradrenaline, and is also selectively concentrated in NAT-expressing tissues and tumours by this process (6, 7).

Targeted therapy of neuroblastoma using [¹³¹I]MIBG has produced encouraging results (long-term remissions and palliation) in patients with resistant disease (8-10). However, the most effective way to use this drug has yet to be defined and, increasingly, [¹³¹I]MIBG is administered in combination with other treatments (11-12). Optimisation of radiation damage induced in target cells can be obtained by rational combination of [¹³¹I]MIBG with radiosensitising agents, and we have previously reported that pretreatment with the topoisomerase I (Topo-I) poison topotecan increased the intracellular concentration of [¹³¹I]MIBG (13). Furthermore, we demonstrated that combinations of [¹³¹I]MIBG and topotecan induced disruption of DNA repair in NAT-expressing cells *in vitro*, supra-additive levels of cytotoxicity, and increased efficacy against NAT-expressing

xenografts *in vivo*. Maximal topotecan-induced radiosensitisation resulted from the administration of the drug simultaneously with, or following [¹³¹I]MIBG. Exposure to topotecan prior to [¹³¹I]MIBG was less effective (14, 15), suggesting that increased [¹³¹I]MIBG uptake due to prior topotecan administration was less important an influence on efficacy as the increased disruption of DNA repair observed in cells treated by combination schedules where topoisomerase I was inhibited concurrently, or following [¹³¹I]MIBG administration.

On the basis of our findings, clinical investigations of MATIN (MIBG And Topotecan In Neuroblastoma) have commenced (16). To date, more than 70 patients in 5 institutions across Europe have received MATIN. Encouraging responses have been observed in a heterogeneous group of patients with relapsed or primary refractory disease. It is our intention to further enhance the effectiveness of MATIN by identifying other clinically relevant drugs which synergise with either topotecan, [¹³¹I]MIBG, or with both agents.

Poly(ADP-ribose) polymerase (PARP-1) is an enzyme involved in DNA repair (17, 18) and it has been demonstrated that PARP-1 inhibition enhanced the efficacy of low dose radiation (19). Furthermore, PARP-1 binds directly to **Topo-I**, leading to increased formation of **Topo-I**-DNA complexes (20). Therefore, we conjectured that PARP-1 inhibition may also influence topotecan-induced toxicity. This study investigated the effects of PARP-1 inhibition on the efficacy of topotecan, [¹³¹I]MIBG and [¹³¹I]MIBG/topotecan combination therapy.

MATERIALS AND METHODS

Drugs

Topotecan (topoisomerase I inhibitor) was purchased from Axxora (UK) Ltd, and PJ34 (PARP-1 inhibitor) from Merck Chemicals Ltd. For *in vitro* experiments, no-carrier-added [¹³¹I]MIBG was provided by Dr S Pimlott, NHS Greater Glasgow and Clyde. For *in vivo* experiments, no-carrier-added [¹³¹I]MIBG was provided by Molecular Insight Pharmaceuticals Inc (Cambridge, Massachusetts, USA).

Cells and Culture Conditions

The following human cell lines were cultured: SK-N-BE(2c), derived from neuroblastoma (21), and UVW/NAT, derived by transfection of the noradrenaline transporter gene into the human glioma cell line UVW (22). UVW/NAT cells were maintained in MEM containing 10% foetal calf serum and 2 mM glutamine. SK-N-BE(2c) cells were maintained in DMEM containing 15% foetal calf serum, non-essential amino acids, 2 mM glutamine. Cell lines were maintained at 37°C in a 5% CO₂ atmosphere. All media and supplements were purchased from Gibco (Paisley, UK). Cell lines were authenticated routinely using the Applied Biosystems AmpF/STR Identifiler Kit (Applied Biosystems UK).

No-carrier-added Synthesis of [¹³¹I]MIBG via Polymer Supported Precursor

For *in vivo* experiments, no-carrier-added [¹³¹I]MIBG was prepared using a solid-phase system where the precursor of [¹³¹I]MIBG was attached to an insoluble polymer via the tin-aryl bond (23, 24). For *in vitro* experiments, no-carrier-added [¹³¹I]MIBG was prepared using a liquid phase system, using the trimethylsilyl precursor (ABX, Germany) (25). The reaction conditions, HPLC purification procedure, and radiochemical yield were as described previously (24).

Determination of Cytotoxicity Following Combination Therapy

In order to investigate the effects of PARP-1 inhibition on [¹³¹I]MIBG/topotecan therapy, initially combinations of topotecan and PJ34 were evaluated using three treatment schedules: topotecan administered 24 h before (1), after (2) or simultaneously with (3) PJ34.

Using similar scheduling, combinations of PJ34 & [¹³¹I]MIBG and triple combinations of PJ34 & [¹³¹I]MIBG/topotecan were also evaluated.. As we have already reported that administration of [¹³¹I]MIBG and topotecan induced supra-additive responses, in the assessment of PJ34, [¹³¹I]MIBG and topotecan 3-drug combinations, PJ34 was administered 24 h before (1), after (2) or simultaneously with (3) [¹³¹I]MIBG and topotecan given simultaneously.

Cytotoxicity was measured by clonogenic assay. Monolayers of cells were cultured in 25cm² flasks (Nunclon Plastics, Denmark). UVW/NAT and SK-N-BE(2c) cells were seeded at 2 x 10⁵ and 4 x 10⁵ cells per flask respectively. After two days, when the cultures were 70% confluent, medium was removed and replaced with fresh medium

containing the appropriate concentration of test drug. Cells were incubated with [¹³¹I]MIBG for 2 hours, after which uptake is maximal (26). Cells were incubated with topotecan and/or PJ34 for 24 hours.

After experimental therapy, cells were washed twice in phosphate buffered saline (PBS), detached by treatment with 0.05 % (v/v) trypsin-EDTA (Gibco, Paisley, UK), counted and seeded, in triplicate, in 60 x 15mm plastic dishes (Nunclon Plastics, Denmark) at 2.5×10^2 cells per dish, for every test concentration. Cultures were incubated at 37°C in 5% CO₂ for 14 days. Colonies were fixed in 100% methanol, and visualised by staining with a solution of 1 % (v/v) Giemsa (BDH Laboratory Supplies) and counted.

Synergy Analysis

The efficacy of the various scheduled combinations was examined according to the method of Chou and Talalay, which is based on the median-effect principle (27). Briefly, from the results of clonogenic assays, dose-effect curves were plotted using the equation $\log[fa/fu] = m\log D - m\log IC_{50}$, where D is the drug dose, fa and fu are, respectively, the fraction of cells affected by drug dose D and the unaffected fraction and IC_{50} is the dose which inhibited 50% of colony formation. From these survival plots, the x-intercept ($\log [IC_{50}]$) and slope m were generated for each treatment. These parameters were used to calculate D , the doses of component agents (and combinations) required to produce various levels of toxicity.

The effectiveness of combination therapy was then assessed by combination-index analysis (27). The toxicities induced by single drugs and

scheduled combinations were investigated using the equation $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$, where $(D)_1$ and $(D)_2$ are the doses of each agent which inhibit x% of cell growth when used in combination and $(Dx)_1$ and $(Dx)_2$ are the doses of each drug, administered as single agents, which inhibit x% of colonies. The resultant numerical values, the combination indices were plotted against toxicity level.

Assessment of Cell Cycle Progression by FACS Analysis.

Cells were plated in 25cm² flasks and exposed to single drug and multi-drug combinations as described above. In order to directly compare the effects on cell cycle progression to the results of clonogenic assay, the same drug concentrations and incubation times were used. Cultures were then trypsinised, counted, washed twice with PBS and resuspended in PBS at a concentration of 1×10^6 cells/ml. Cells were fixed by treatment with 75% (v/v) ethanol for 1 h at 4°C. Fixed cells were washed twice with PBS and resuspended in 1ml PBS containing 50 mg/ml propidium iodide (Sigma chemicals, Dorset UK) and 5 µg/ml RNase A (Qiagen Ltd. W. Sussex, UK). Cells were stained for 3 h at 4°C before flow cytometry, using a FACScan analyser (Becton Dickinson Systems, Cowley, UK). Data were analysed using BD CellQuest™ Pro software, version 5.1.1.

Assessment of Double-strand (ds) DNA Breaks by H2A.X Phosphorylation

Phosphorylation of histone H2A.X at serine 139 (γ H2A.X) was assessed using the H2A.X Phosphorylation Assay Kit (Millipore, Herts.

UK). Briefly, cells were seeded and drug-treated, as described above. Immediately following treatment, when DNA damage was expected to be maximal, cells were trypsinised, counted, washed twice with PBS and fixed by addition of formaldehyde/methanol for 20 min at 4°C. Cells were then washed three times with PBS and resuspended, at a concentration of 2×10^6 cells/ml, in permeabilization solution (5% saponin, 100 mM HEPES pH 7.4, 1.4 M NaCl, 25 mM CaCl₂) containing anti-phospho-histone H2A.X (Ser139)-FITC conjugate for 20 min at 4°C. Fluorescence was measured using a FACScan analyser (Becton Dickinson Systems, Cowley, UK). Data were analysed using BD CellQuest™ Pro software, version 5.1.1.

PARP-1 Assay

Cellular PARP-1 activity was assessed using the commercially available HT Universal colorimetric assay kit (AMS Biotechnology Ltd, Abingdon UK), according to the manufacturers instructions. Briefly, following drug treatment as described above, PARP-1 activity was assayed by incorporation of biotinylated poly(ADP-ribose) onto histone proteins, followed by incubation with streptavidin-horseradish peroxidase and TACS-sapphire colorimetric substrate. Absorbance at 450nm was measured using a Sunrise plate reader (Tecan UK Ltd, reading UK), with Magellan CE software (v 5.04).

Experimental Animals.

Six-week-old female, congenitally athymic nude mice of strain MF1 nu/nu were obtained from Charles River plc (Kent, United Kingdom).

Experiments were carried out in accordance with the UK Co-ordinating Committee on Cancer Research guidelines (28).

In Vivo Investigations

SK-N-BE(2c) and UVW/NAT xenografts were established and analysed as previously described (14, 15). Briefly, SK-N-BE(2c) xenografts were established by intrasplenic injection of 3×10^6 exponentially growing cells. Following the growth of tumours in the spleen and liver, animals were euthanized and tumour fragments 2-3 mm in diameter were then implanted subcutaneously in the subcostal flanks of other nude mice. Experimental therapy commenced 17 days after tumour implantation when tumours had reached approximately 10 mm diameter (500 mm^3). UVW/NAT xenografts were established by subcutaneous injection of 2×10^6 cells. Experimental therapy was initiated 9 days later, when tumor volume was approximately 60 mm^3 .

Groups of 6 mice with SK-N-BE(2c) or UVW/NAT tumours were randomized into six treatment groups which received, by intraperitoneal (i.p.) injection, (a) saline (control), (b) 20 mg/kg PJ34 alone, (c) 18 MBq of [^{131}I]MIBG + 1.75 mg/kg of topotecan (SK-N-BE(2c)), or 5 MBq of [^{131}I]MIBG + 0.825 mg/kg of topotecan (UVW/NAT) or (d) PJ34 given simultaneously with [^{131}I]MIBG/topotecan.

Experimental xenografts were measured with calipers immediately prior to treatment and every 2-3 days thereafter. Measurements were converted to an approximate volume on the assumption of ellipsoidal geometry as previously described (14, 15).

For each treatment group, the mean time taken for a 2-fold (T_2) (SK-N-BE(2c)) or 10-fold (T_{10}) (UVW/NAT) increase in tumours was calculated. Tumour cure was defined as the failure of experimental xenografts to increase in size over the experimental time course.

Statistical Analysis

Unless otherwise stated, experimental results are expressed as means and standard deviations of three separate experiments, carried out in triplicate. Statistical analyses were carried out using GraphPad Prism software, version 4.03 (GraphPad software Inc.). One-way analysis of variance (ANOVA) was used to compare, between treatments, formation of dsDNA damage and PARP-1 activity. Post hoc testing used Bonferroni's correction for multiple comparisons. Differences in tumour growth between experimental therapy groups were assessed by the Kruskal-Wallis test, followed by post hoc testing by the Mann-Whitney U test. With Bonferroni's correction, $P < 0.01667$ was considered significant.

RESULTS

Cytotoxicity of 2-drug Combination Therapy

For both cell lines, the efficacy of topotecan and PJ34 as single agents was assessed, and results were plotted graphically, according to the median-effect principle (Supplementary Figure 1A). On the basis of single drug toxicity, a fixed ratio of topotecan : PJ34 was used in subsequent analyses of alternative combination schedules The topotecan (nM): PJ34

(μM) ratios were 8.8 : 31.97 for SK-N-BE(2c) cells; and 10 : 29.1 for UVW/NAT cells. The median-effect plots for alternative topotecan/PJ34 combination schedules are shown in Supplementary Figure 1B.

Combination-index analysis of topotecan & PJ34 treatments in (A) SK-N-BE(2c) and (B) UVW/NAT cells are shown in Figure 1. Both cell lines were resistant to doses of combinations of topotecan & PJ34 which induced low levels of toxicity. Schedule (3) (topotecan & PJ34 simultaneously) was the most effective treatment, inducing supra-additive responses in both lines. In SK-N-BE(2c) cells, supra-additive responses were also observed following schedule (2) treatment (topotecan after PJ34, but not schedule (1) (topotecan before PJ34). All three schedules induced supra-additivity in UVW/NAT cells at high levels of toxicity.

Dose-responses for PJ34 and [^{131}I]MIBG as single agents are shown in Supplementary Figure 2A. On the basis of these results, the ratios of PJ34 (μM): [^{131}I]MIBG (MBq/ml) used in subsequent combination studies were 31.97 : 1.29 for SK-N-BE(2c) cells; and 29.1 : 2.76 for UVW/NAT cells.. The effects of scheduled combinations are shown in Supplementary Figure 2B. The resultant combination-index analyses of (A) SK-N-BE(2c) and (B) UVW/NAT cells are shown in Figure 2.

Both cell lines were resistant to doses of combinations of PJ34 & [^{131}I]MIBG which induced low levels of toxicity. Schedule (3) (PJ34 & [^{131}I]MIBG simultaneously) induced supra-additive responses in both cell lines. In SK-N-BE(2c) cells, schedule (1) (PJ34 before [^{131}I]MIBG) induced an additive response, whereas schedule (2) (PJ34 after [^{131}I]MIBG) induced infra-additive toxicity. In UVW/NAT cells, supra-additive

responses were also observed following administration of schedule (2), whereas schedule (1) induced infra-additive toxicity.

Cytotoxicity of 3-drug Combination Therapy

Dose-responses for PJ34 as a single agent and [¹³¹I]MIBG/topotecan 2-drug combinations are shown in Supplementary Figure 3A. On the basis of these results, the ratios of PJ34 (μM) : [¹³¹I]MIBG (MBq/ml) : topotecan (nM) used in subsequent 3-drug combination studies were 31.97 : 1.29 : 8.8 for SK-N-BE(2c) cells; and 29.1 : 2.76 : 10 for UVW/NAT cells. Therefore 1 arbitrary dose unit (au) contained 0.76 μM PJ34, 0.03 MBq/ml [¹³¹I]MIBG and 0.21 nM topotecan or 0.69 μM PJ34, 0.07 MBq/ml [¹³¹I]MIBG and 0.24 nM topotecan for SK-N-BE(2c) and UVW/NAT cells respectively.

The effects of scheduled triple combinations are shown in Supplementary Figure 3B. The resultant combination-index analyses of (A) SK-N-BE(2c) and (B) UVW/NAT cells are shown in Figure 3.

Supra-additive toxicity was observed in SK-N-BE(2c) cells following all three schedules at every level of toxicity assessed. Only schedule (3) (PJ34 & [¹³¹I]MIBG/topotecan simultaneously) induced enhanced efficacy in UVW/NAT cells. Schedule (2) (PJ34 after [¹³¹I]MIBG/topotecan) induced an additive response in UVW/NAT cells, whereas schedule (1) (PJ34 before [¹³¹I]MIBG/topotecan) was antagonistic.

Cell Cycle Redistribution

Cell cycle redistribution induced by PJ34 in SK-N-BE(2c) and UVW/NAT cells is shown in Table 1. The effects of triple combination

treatments upon cell cycle phases are shown in Table 2 (SK-N-BE(2c)) and Table 3 (UVW/NAT). PJ34 as a single agent induced G₂/M arrest. Likewise, **all three scheduled** combinations of PJ34 & [¹³¹I]MIBG/topotecan caused an increase in the number of cells in G₂/M.

γH2A.X Analysis of dsDNA Damage

The effects of 3-drug administration on formation of dsDNA breaks are shown in Figure 4A. Cells treated with PJ34 & [¹³¹I]MIBG/topotecan combinations displayed increased phosphorylation of H.2AX foci compared to untreated controls. In SK-N-BE(2c) cells, all treatment schedules induced a **28-45-fold** increase in dsDNA damage. Analysis of variance demonstrated no significant difference between the potency of alternative combination schedules. However, in Uvw/NAT cells, administration of alternative schedules gave rise to various levels of DNA damage. Schedule (1) induced a 2-fold increase in γH2A.X foci, whereas schedule (3) induced a 60-fold increase. Schedule (2) induced a 20-fold increase in H2A.X phosphorylation. Analysis of variance demonstrated significant variation between the responses induced by alternative schedules of delivery and in post-hoc testing, Schedule (3) induced significantly higher levels of γH2A.X phosphorylation than either of the other treatment schedules (p < 0.01667).

PARP-1 Activity

The effects of (i) PJ34 treatment and (ii) [¹³¹I]MIBG/topotecan 2-drug therapy on PARP-1 activity in SK-N-BE(2c) and Uvw/NAT cells are

shown in Figure 4B. PJ34 induced a dose-dependent reduction in PARP-1 activity compared to untreated controls. SK-N-BE(2c) cells were more sensitive to PJ34 than UVW/NAT cells. The dose which reduced PARP-1 activity by 50% (EC_{50}) was 8.8 μ M and 14.6 μ M, in SK-N-BE(2c) cells and UVW/NAT cells respectively.

In SK-N-BE(2c) cells, compared with untreated controls, administration of [131 I]MIBG/topotecan induced a reduction in PARP-1 activity at concentrations less than, or equal to 21.03 au. [131 I]MIBG/topotecan also reduced PARP-1 activity in UVW/NAT cells at concentrations less than, or equal to 20.93 au. As with PJ34, SK-N-BE(2c) cells were more sensitive to [131 I]MIBG/topotecan than UVW/NAT cells (EC_{20} values were 6.8 au and 14.5 au for SK-N-BE(2c) and UVW/NAT cells respectively). However, in both cell lines, following administration of the highest administered dose of [131 I]MIBG/topotecan (31.55 au for SK-N-BE(2c) cells; 31.39 au for UVW/NAT cells), 100% recovery of PARP-1 activity was observed, suggesting an adaptive response to [131 I]MIBG/topotecan-induced disruption of PARP-1 function.

The effects of PJ34 & [131 I]MIBG/topotecan combination therapy on PARP-1 activity in (i) SK-N-BE(2c) and (ii) UVW/NAT cells are shown in Figure 4C. 3-drug therapy reduced PARP-1 activity in SK-N-BE(2c) cells compared to untreated controls. Schedule (3) (simultaneous administration) was the most effective schedule. However, unlike [131 I]MIBG/topotecan 2-drug therapy, there was no recovery in PARP-1 activity at higher doses in SK-N-BE(2c) cells. In UVW/NAT cells, administration of Schedules (2) & (3) (PJ34 simultaneously with, or after [131 I]MIBG/topotecan) also induced

a reduction in PARP-1 activity. Again, there was no evidence of a recovery of PARP-1 function at higher doses. However, Schedule (1) (PJ34 before [¹³¹I]MIBG/topotecan) had no effect on PARP-1 activity. This suggests that inhibition of PARP-1 function by PJ34 was not only reversed after removal of the drug, but provoked resistance to subsequent [¹³¹I]MIBG/topotecan-induced inhibition of PARP-1 function in this cell line.

***In Vivo* Investigations**

None of the animals in this study showed signs of distress. Figure 5 shows the effect, on the growth of (A) SK-N-BE(2c) and (B) UVW/NAT tumor xenografts, of the administration of PJ34 or [¹³¹I]MIBG/topotecan either alone or in combination. Tumor growth times and cure rates for SK-N-BE(2c) and UVW/NAT xenografts are presented in Table 4.

For both xenograft models, overall differences in the effectiveness of the different treatments were highly significant (Kruskal-Wallis test, $P < 0.005$). Single treatment with PJ34 did not significantly affect tumour growth. Treatment with [¹³¹I]MIBG/topotecan or PJ34 in combination with [¹³¹I]MIBG/topotecan significantly delayed SK-N-BE(2c) and UVW/NAT tumor growth compared to PBS treated controls ($P < 0.01667$). Furthermore, 3-drug therapy also significantly increased the delay in tumour growth compared to [¹³¹I]MIBG/topotecan double combinations in both *in vivo* models ($P < 0.01667$). No SK-N-BE(2c) tumors were cured during the course of these experiments. In contrast, 60% of UVW/NAT tumors were cured by [¹³¹I]MIBG/topotecan treatment, whereas 3-drug treatment cured all UVW/NAT tumors.

DISCUSSION

Previously, we reported that topotecan (**topoisomerase I inhibitor**) synergised with [¹³¹I]MIBG. The present study indicated that PJ34 enhanced the efficacy of topotecan and [¹³¹I]MIBG *in vitro*, and [¹³¹I]MIBG/topotecan combination therapy *in vitro* and *in vivo*. Enhanced [¹³¹I]MIBG/topotecan efficacy was associated with disruption of PARP-1 activity, increased formation of dsDNA breaks and G₂/M cell cycle arrest.

Enhanced efficacy was most likely caused by disruption of DNA damage repair pathways. PARP-1 is involved in the repair of single-strand DNA breaks through the base excision repair (BER) pathway (29) and may also be involved in repair of double-strand breaks, through the homologous recombination (HR) pathway (30). Furthermore, we have previously demonstrated that topotecan and [¹³¹I]MIBG, either alone or in combination, induced G₂-phase cell cycle arrest (15). In this study, treatment with PJ34 either as a single agent, or in combination with [¹³¹I]MIBG/topotecan, also caused G₂ arrest. Cells in G₂- and M-phase are more radiosensitive than cells in other phases of the cell cycle (31). Thus cell cycle redistribution induced by treatment with PJ34 & [¹³¹I]MIBG/topotecan **combinations** probably contributed to the enhanced efficacy of [¹³¹I]MIBG.

Following topotecan treatment, **Topo-I** becomes strongly associated with DNA via stabilisation of **Topo-I**-DNA complexes, leading to stalled DNA replication. PARP-1-mediated ADP-ribosylation of **Topo-I** reprograms the trapped enzyme to remove itself from cleaved DNA (32). PARP-1 also collaborates with Mre11, a core subunit of the

Mre11/Rad50/Nbs1 damage recognition complex, to promote replication fork restart after release from replication blocks (30). Thus, by counteracting topoisomerase I-induced DNA damage, PARP-1 activity acts as a positive regulator of genomic stability in eukaryotic cells.

It has previously been demonstrated that following the induction of DNA damage by X-ray, UV and gamma irradiation, binding of PARP-1 to Topo-I induces a rapid sequestration of Topo-I onto the sites of the DNA lesions (33-35). Furthermore, Topo-I-mediated unwinding of supercoiled DNA is reduced following irradiation, possibly by abrogation of Topo-I catalytic activity (36, 37), or reduced longevity of Topo-I–DNA complexes (38). This effect appears to be due to PARP-1-induced ADP-ribosylation of Topo-I, and is prevented by the addition of PARP-1 inhibitors (36, 37),

Therefore, in cells treated with combinations of [¹³¹I]MIBG and topotecan, inhibition of PARP-1 activity by PJ34 leads to the simultaneous generation of multiple effects. Deregulation of Topo-I function via ADP-ribosylation and prevention of removal of topotecan-mediated aberrant Topo-I-DNA adducts will enhance the efficacy of the Topo-I poison, while disruption of BER and potentially, HR mechanisms of repair would increase the effects of [¹³¹I]MIBG-induced DNA damage.

Treatment with [¹³¹I]MIBG/topotecan induced a reduction in PARP-1 activity, reaching a nadir at a combination dose equivalent to 21.03 au and 20.93 in SK-N-BE(2c) and UVW/NAT cells respectively. It has long been recognised that MIBG is an inhibitor of mono-ADP-ribosylation (39). Furthermore, it has recently been suggested that, in the absence of DNA damage, PARP-1 function is regulated by the mono-ADP-ribosyl

polymerase activity of the related enzyme PARP-3 (40). Therefore, **one possible explanation for these findings is that** reduced PARP-1 function following administration of low doses of [¹³¹I]MIBG/topotecan **is** due to MIBG-induced inhibition of PARP-3 regulation of PARP-1. However, following administration of doses of [¹³¹I]MIBG/topotecan greater than 21.03 au (SK-N-BE(2c)) or 20.93 au (UVW/NAT), PARP-1 function recovered in and both cell lines. Increased DNA damage was observed with increasing dose of 3-drug therapy. Taken together, these results suggest that **if, as speculated, MIBG inhibits PARP-3, increasing DNA damage may induce activation of PARP-1** via a PARP-3-independent pathway, leading to **the observed** recovery of PARP-1 function **after high-dose** [¹³¹I]MIBG/topotecan treatment.

UVW/NAT cells treated with PJ34 prior to [¹³¹I]MIBG/topotecan displayed no disruption of PARP-1 function. PJ34 induced a reduction of PARP-1 activity, which was restored by removal of the drug. The subsequent addition of [¹³¹I]MIBG/topotecan had no effect on PARP-1 function, suggesting that Uvw/NAT cells were primed to resist [¹³¹I]MIBG/topotecan-induced disruption of PARP-1 activity by PJ34 pre-treatment. It is possible that the recovery of PARP-1 function in PJ34 pre-treated cells may be induced by a PARP-3-independent pathway which would be unaffected by PARP-3 inhibition by MIBG. This suggests that the efficacy of PJ34/[¹³¹I]MIBG/topotecan treatment may be affected by alternative PARP-1 activation pathways and warrants further study.

While the involvement of PARP-1 in repair of dsDNA damage is as yet unclear, this present study does suggest that PARP-1 activity is involved in

this process in some way. SK-N-BE(2c) cells, which exhibited reduced PARP-1 function in this phase, also displayed increased generation of dsDNA damage following 3-drug treatment, leading to supra-additive cytotoxicity. Conversely, UVW/NAT cells treated with PJ34 prior to [¹³¹I]MIBG/topotecan exhibited normal PARP-1 function and DNA damage was negligible, suggesting less inhibition of the repair of dsDNA breaks, leading to infra-additive toxicity in cells treated by this schedule. Combination therapy also induced G₂/M arrest, where the predominant dsDNA damage repair pathway is HR. Therefore it is possible that PARP-1 may play a role in HR, however involvement with non-homologous end joining (NHEJ) cannot be discounted.

PJ34 alone induced cytotoxicity and G₂/M arrest. Therefore, non-target effects may influence overall response. This will be addressed by further mechanistic studies, utilising PJ34 and also second- and third-generation inhibitors with greater PARP-1 specificity.

Previously, we demonstrated that [¹³¹I]MIBG/topotecan combination therapy significantly inhibited SK-N-BE(2c) and UVW/NAT tumor growth *in vivo* (14, 15). In this study, while PJ34 treatment had no effect on tumour growth, administration of PJ34 concurrently with [¹³¹I]MIBG/topotecan significantly delayed the growth of SK-N-BE(2c) and UVW/NAT xenografts compared to [¹³¹I]MIBG/topotecan.

CONCLUSION

This study indicates that inhibition of PARP-1 has the potential to increase the efficacy of [¹³¹I]MIBG/topotecan combination therapy, by increasing

radiosensitivity and disrupting DNA repair. Taking into account the responses observed both *in vitro* and *in vivo*, this study suggests that enhancement of [¹³¹I]MIBG/topotecan efficacy may be best achieved by the simultaneous inhibition of PARP-1 function. Elucidation of the basis for resistance following pre-treatment with PJ34 may allow further refinements to this combination and are worthy of investigation.

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FIGURE LEGENDS

Figure 1

Combination index analysis of PJ34/topotecan combination treatment in (A) SK-N-BE(2c) and (B) UVW/NAT cells. Based on the results shown in Supplementary Figure 1, SK-N-BE(2c) were treated with topotecan and PJ34 in a ratio of 8.8 : 31.97. UVW/NAT were treated with PJ34 and topotecan in a ratio of 10 : 29.1. Data are means and standard deviations of triplicate determinations from three experiments. $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergism, additivity and antagonism respectively.

Figure 2

Combination index analysis of PJ34/ ^{131}I]MIBG combination treatment in (A) SK-N-BE(2c) and (B) UVW/NAT cells. Based on the results shown in Supplementary Figure 2, SK-N-BE(2c) were treated with PJ34 and ^{131}I]MIBG in a ratio of 31.97 : 1.29. UVW/NAT were treated with PJ34 and ^{131}I]MIBG in a ratio of 29.1 : 2.76. Data are means and standard deviations of triplicate determinations from three experiments. $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergism, additivity and antagonism respectively.

Figure 3

Combination index analysis of PJ34/ ^{131}I]MIBG/topotecan combinations in (A) SK-N-BE(2c) and (B) UVW/NAT cells. Based on the results shown in Supplementary Figure 3, SK-N-BE(2c) were treated with PJ34, ^{131}I]MIBG and topotecan in a ratio of 31.97 : 1.29 : 8.8. UVW/NAT were treated with PJ34, ^{131}I]MIBG and topotecan in a ratio of 29.1 : 2.76 : 10. Data are

means and standard deviations of triplicate determinations from three experiments. $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergism, additivity and antagonism respectively.

Figure 4

A. γ H2A.X analysis of dsDNA damage in (i) SK-N-BE(2c) and (ii) UVW/NAT cells. **B.** PARP-1 activity in SK-N-BE(2c) and UVW/NAT cells following (i) PJ34 and (ii) [131 I]MIBG/topotecan treatment. **C.** PARP-1 activity in (i) SK-N-BE(2c) and (i) UVW/NAT cells following PJ34 & [131 I]MIBG/topotecan combination treatment. The ratios of PJ34 and [131 I]MIBG/topotecan used in 2-drug and 3-drug combinations were as described in figure 3. Data are means and standard deviations of triplicate determinations from three experiments.

Figure 5

The effects of PJ34 and [131 I]MIBG/TPT on **(A)** SK-N-BE(2c) and **(B)** UVW/NAT xenografts. Each treatment group consisted of six animals. Mice bearing SK-N-BE(2c) xenografts were treated with either 20mg/kg PJ34, 18MBq [131 I]MIBG + 1.75mg/kg topotecan or combinations of PJ34 and [131 I]MIBG/topotecan. UVW/NAT-bearing animals were treated with either 20mg/kg PJ34, 5MBq [131 I]MIBG + 0.875 mg/kg topotecan or 3-drug combinations.