[^{131}I]*meta*-Iodobenzylguanidine and Topotecan Combination Treatment of Tumors Expressing the Noradrenaline Transporter

Anthony G. McCluskey,[1] Marie Boyd,[1] Susan C. Ross,[1] Emilio Cosimo,[1] Anne Marie Clark,[1] Wilson J. Angerson,[2] Mark N. Gaze,[3] and Robert J. Mairs[1]

Abstract

Purpose: Both [^{131}I]*meta*-iodobenzylguanidine ([^{131}I]MIBG) and the topoisomerase I inhibitor topotecan are effective as single-agent treatments of neuroblastoma. The aim of this study was to investigate the efficacy of [^{131}I]MIBG in combination with topotecan *in vitro* and *in vivo*.

Experimental Design: The cell lines used were SK-N-BE(2c) (human neuroblastoma) and UVW/NAT (glioma cell line transfected with the noradrenaline transporter gene). Three different treatment schedules were assessed: topotecan given before (schedule 1), after (schedule 2), or simultaneously (schedule 3) with [^{131}I]MIBG. DNA strand breakage was evaluated by comet assay, and cytotoxicity was determined by clonogenic survival. Efficacy was also measured by growth delay of tumor xenografts in nude mice.

Results: Combination schedules 2 and 3 caused more cytotoxicity than schedule 1. Similarly, significant DNA damage was observed following treatment schedules 2 and 3 ($P < 0.005$) but not schedule 1. The mean number of days for a doubling in volume of SK-N-BE(2c) tumors and a 10-fold increase in volume of UVW/NAT tumors were 10.4 and 18.6 (untreated), 19.7 and 25.3 (topotecan alone), 22.8 and 31.9 ([^{131}I]MIBG alone), 26.3 and 37.1 (combination schedule 1), 34.3 and 49.7 (combination schedule 2), and 53.2 and >71 (combination schedule 3), respectively. The highest rate of cure of both xenografts was observed following treatment with combination schedule 3.

Conclusions: The combination of topotecan and [^{131}I]MIBG compared with either treatment alone gave rise to greater than additive DNA damage, clonogenic cell kill, and tumor growth delay. These effects were dependent on the scheduling of the two agents.

Despite progress in the treatment of several childhood cancers, little improvement has been achieved in the cure rate of neuroblastoma patients with advanced disease. [^{131}I]*meta*-iodobenzylguanidine ([^{131}I]MIBG), an analogue of adrenergic neuron blockers (1), is selectively concentrated in tumors of neural crest origin, such as neuroblastoma (2) and phaeochromocytoma (3), via the noradrenaline transporter (NAT; ref. 4). When appropriately radiolabeled, [^{131}I]MIBG can be used for diagnostic scintigraphy or radionuclide therapy, resulting in specific irradiation of the target tumor cells with relative sparing of normal tissues. Targeted therapy using [^{131}I]MIBG has achieved long-term remissions and palliation in neuroblastoma patients with resistant disease (5–7). However, the most effective way to use this tumor-targeting drug has yet to be defined; increasingly, [^{131}I]MIBG is given in combination with other therapies (8) in attempts to realize its full potential (9).

The topoisomerase I inhibitor topotecan (10), an analogue of camptothecin, is effective as a single treatment of refractory neuroblastoma (11), but perhaps more importantly it has also been shown to act as a radiosensitizer *in vitro* (12). Therefore, it may be possible to augment the potency of [^{131}I]MIBG treatment of neuroblastoma by incorporating this form of targeted radiotherapy with topotecan.

Despite the large number of experimental therapy studies of camptothecin derivatives combined with external beam irradiation, there is no consensus with respect to the optimal scheduling of these modalities (13–18). Similarly, the most favorable sequencing of [^{131}I]MIBG and topotecan treatments is difficult to predict because of physical and biological factors specific to targeted radionuclide therapy. For example, it has been shown that the treatment of tumor cells with a variety of metabolic effectors resulted in increased accumulation of subsequently given [^{131}I]MIBG (19, 20). Conversely, cell kill resulting from cytotoxic drug treatments could also reduce the capacity of tumors to actively concentrate [^{131}I]MIBG with subsequent decrease in the magnitude of the radiation cross-fire component of cell kill (21).

This study aimed to assess *in vitro* (by median-effect analysis) and *in vivo* the schedule of [^{131}I]MIBG and

Authors' Affiliations: [1]Targeted Therapy Group and Department of Child Health, University of Glasgow, Cancer Research UK Beatson Laboratories; [2]Department of Surgery, University of Glasgow, Royal Infirmary, Glasgow, United Kingdom; and [3]University College London Hospitals, NHS Foundation Trust, Middlesex Hospital, London, United Kingdom

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Requests for reprints: Robert J. Mairs, Department of Radiation Oncology, University of Glasgow, Cancer Research UK Beatson Laboratories, Garscube Estate, Glasgow G61 1BD, United Kingdom. Phone: 44-41-330-4126; Fax: 44-41-330-4127; E-mail: r.mairs@beatson.gla.ac.uk.

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topotecan that would produce optimal efficacy, to determine the influence of inhibition of DNA repair on the radiosensitization effect of topotecan, and to evaluate the toxicity of [^{131}I]MIBG/topotecan combination treatment to bone marrow, the dose-limiting tissue for both agents.

Materials and Methods

Cells and culture conditions. The following human tumor cell lines were cultured: SK-N-BE(2c) (NAT-expressing cell line derived from neuroblastoma; refs. 20, 22) and UVW/NAT (derived by transfection of the bovine *NAT* gene into the human glioma cell line UVW; ref. 23). Cells were maintained in MEM (UVW/NAT) or RPMI 1640 [SK-N-BE(2c)] containing 10% fetal bovine serum and glutamine (2 mmol/L) at 37°C in a 5% CO_2 atmosphere. All media and supplements were purchased from Invitrogen (Paisley, United Kingdom). Topotecan was generously provided by GlaxoSmithKline Pharmaceuticals (Pennsylvania, PA).

Synthesis of [^{131}I] meta-iodobenzylguanidine. No-carrier-added [^{131}I]MIBG was prepared using a solid-phase system where the precursor of [^{131}I]MIBG was attached to an insoluble polymer via the tin-aryl bond (24). The reaction conditions, HPLC purification procedure, and radiochemical yield were as described previously (25).

[^{131}I] meta-iodobenzylguanidine uptake in cell lines. The ability of cell lines to accumulate [^{131}I]MIBG was assessed as described previously (26).

Clonogenic assay. SK-N-BE(2c) and UVW/NAT cells were seeded in 25 cm^2 flasks at 0.5×10^5 per flask. After 2 days, when the cultures were 70% confluent, medium was removed and replaced with fresh medium alone or medium containing a range of concentrations of topotecan alone, [^{131}I]MIBG alone, or various combinations of the two agents. Three different combination treatment schedules were assessed: topotecan given 24 hours before [^{131}I]MIBG (schedule 1), topotecan given 24 hours after [^{131}I]MIBG (schedule 2), and topotecan and [^{131}I]MIBG given simultaneously (schedule 3). Cells were incubated with [^{131}I]MIBG for 2 hours, after which uptake is maximal (26). The duration of incubation in the presence of topotecan was 24 hours. This exposure has been shown previously to result in optimal sensitization to external beam irradiation (16). After drug treatment, medium was removed and the cells were washed thrice with NaCl solution, suspended by treatment with trypsin and counted using a hemocytometer. For every treatment, 1×10^3 cells were seeded, in triplicate, in 25 cm^2 flasks (Nunclon Plastics, Roskilde, Denmark) in 10 mL fresh medium. The cultures were then incubated at 37°C in 5% CO_2. After 10 to 14 days, medium was removed and the colonies were fixed and stained with carbol fuchsin (R.A. Lamb, Middlesex, United Kingdom) before counting.

Analysis of interaction between topotecan and [^{131}I] meta-iodobenzylguanidine. The cytotoxic interaction between topotecan and [^{131}I]MIBG was examined according to the method of Chou and Talalay (27), which is based on the median-effect principle (28). Initially, exponentially growing cells were treated with each agent alone to determine the dose required for 50% clonogenic cell kill (IC_{50}). Based on the IC_{50} concentrations, cells were subsequently treated with serial dilutions of combinations of topotecan and [^{131}I]MIBG according to schedules 1 to 3 using a fixed dose ratio of topotecan to [^{131}I]MIBG, so that the proportional contribution of each drug in the mixtures would be the same at all treatment intensities. SK-N-BE(2c) cells were treated with topotecan and [^{131}I]MIBG in a fixed ratio so that 1 dose unit was equivalent to 0.33 ng/mL topotecan + 18 μCi/mL [^{131}I]MIBG. UVW/NAT cells were treated with topotecan and [^{131}I]MIBG in a fixed ratio so that 1 dose unit corresponded to 0.5 ng/mL topotecan + 13.5 μCi/mL [^{131}I]MIBG.

The effectiveness of combinations of topotecan and [^{131}I]MIBG was quantified by determining a combination index (CI) at various levels of cytotoxicity for each schedule. As the method of interaction was unknown, CIs were calculated assuming both mutual exclusivity (where the drugs have similar mechanisms of action) and mutual nonexclusivity (where the drugs have dissimilar mechanisms of action; ref. 27). CI < 1, CI = 1, and CI > 1 indicate synergism, additivity, and antagonism, respectively.

Comet assay. To evaluate the influence of topotecan on the repair of radiation damage to DNA, fragmentation of DNA was determined using the Trevigen CometAssay Single-Cell Electrophoresis Assay kit (Trevigen, Inc., Gaithersburg, MD) according to the manufacturer's instructions. DNA was stained with 1× SYBR Green solution (Trevigen) and examined by fluorescence microscopy (435-500 nm) using a Zeiss Axiovert inverted microscope (Carl Zeiss Ltd., Welwyn Garden City, United Kingdom). Images were captured by a Zeiss MC 100 SPOT camera and saved by Axiovision 3.0.6.1 software. One hundred twenty cells per treatment were analyzed using a computer-based image analysis system (NIH Image 1.57 software). The amount of DNA fragmentation was expressed as the tail moment, which combined a measurement of the length of the DNA migration with the relative amount of DNA therein (29).

For both SK-N-BE(2c) and UVW/NAT cells, comet assay was done immediately after drug treatment and 24 hours later. Cellular capacity to repair DNA damage was determined by comparison of tail moment at these two time points by the Mann-Whitney *U* test.

Experimental animals. Six-week-old female, congenitally athymic nude mice of strain MF1 *nu/nu* were obtained from Charles River plc (Kent, United Kingdom). *In vivo* experiments were carried out in accordance with the UK Co-ordinating Committee on Cancer Research guidelines on experimental neoplasia in animals (30).

Xenografts. Tumor growth was established by intrasplenic injection of 3×10^6 exponentially growing SK-N-BE(2c) cells as described by Rutgers et al. (31). These xenografts could not be established by s.c. injection of SK-N-BE(2c) cells. Following the growth of tumors in the spleen and liver, animals were euthanized, and tumor fragments (2-3 mm in diameter) were then implanted s.c. in the subcostal flanks of other nude mice. Mice were used for experimental therapy 17 days after tumor implantation when the s.c. tumors had reached ~10 mm in diameter (~500 mm^3).

Xenografts were also established in nude mice by s.c. injection of 2×10^6 UVW/NAT cells as described previously (25). Experimental therapy was initiated 9 days later, at which time tumor volume was ~60 mm^3. The use of two different size classes of experimental tumor enabled our comparison of the effectiveness of the combination treatment applied to different target volumes. To monitor potential toxicity, experimental animals were examined daily for signs of distress using standard guidelines (32).

Effect of topotecan pretreatment on the biodistribution of [^{131}I] meta-iodobenzylguanidine. Twenty-four hours before administration of [^{131}I]MIBG, the mice received by i.p. injection 1.75 mg/kg topotecan or the equivalent volume of NaCl solution. One hour before [^{131}I]MIBG injection, tumor-bearing mice were injected i.p. with 1 mL of a 0.1% (w/v) potassium iodide solution to diminish thyroid uptake of radioiodine. The animals then received by i.p. injection 27 μCi [^{131}I]MIBG. After 24 hours, mice were euthanized. Samples of femur contents, tumor, heart, lung, adrenal glands, muscle, kidney, thyroid, and liver were excised and weighed and the associated radioactivity was measured in an automated gamma counter (Packard Biosciences Ltd., Berks, United Kingdom).

Therapy experiments. Twelve mice with SK-N-BE(2c) or UVW/NAT tumors were used for each treatment regimen. The mice were randomized into six treatment groups that received by i.p. injection NaCl solution (control), 486 μCi [^{131}I]MIBG alone [SK-N-BE(2c) tumors], 270 μCi [^{131}I]MIBG alone (UVW/NAT tumors), 1.75 mg/kg topotecan alone, or combinations of the two agents. Three treatment schedules were assessed: topotecan given 24 hours before, after, or

simultaneously with [^{131}I]MIBG. The dose of [^{131}I]MIBG given to the mice was shown previously by us to induce significant delay of the growth but incomplete sterilization of SK-N-BE(2c) (33) or UVW/NAT (25) xenografts.

Subcutaneous tumors were measured with calipers immediately before treatment and every 2 or 3 days thereafter. On the assumption of ellipsoidal geometry, diameter measurements were converted to an approximate volume by multiplying half the longest diameter by the square of the mean of the two shorter diameters (34). Mice whose xenograft volume reached 1,900 mm^3 were euthanized.

To enable the estimation of the time required for a 2- or 10-fold increase in size, the tumor volumes against time were plotted for each animal. In the growth/regrowth phase, these profiles were log linear. Separate log-linear regressions were fitted to each animal's data. For each treatment group, the mean time taken to reach 2 times [T_2, SK-N-BE(2c) xenografts] or 10 times (T_{10}, UVW/NAT xenografts) the tumor volume at the initiation of treatment was calculated to allow comparison among treatment groups. Cure was defined as the failure of tumors to increase in size over the experimental time course.

For the purpose of comparing antitumor efficacies of the various treatment combinations, taking into account both growth delay and cure rate, cured tumors were assigned a common T_2 or T_{10} that was higher than that observed in any regrowing tumor (i.e., 71 days). The Kruskal-Wallis test and the Mann-Whitney *U* test were used for overall and pairwise group comparisons, respectively. Because of the high prevalence of tied data associated with cured tumors, a Monte Carlo technique was used to determine significance levels.

Mice were housed and manipulated in a designated category 2, lead-lined, radioisotope suite within the preclinical unit. Disposal of carcasses and bedding was by means of a designated macerator. Animals were handled and bedding was changed within a category 2 hood to minimize the hazard of radioactive aerosols. Tumor measurements were done within a lead-impregnated γ-cabinet (Camlab, Cambridge, United Kingdom) within the hood to minimize exposure to handlers. A lead apron was worn during the handling of radioactive animals. The examination of radioactivity monitoring badges, worn by the experimenters, indicated negligible exposure.

Assessment of marrow toxicity. Separate groups of six mice were used for the determination of possible adverse effects on bone marrow of the various combinations of [^{131}I]MIBG and topotecan. One, 2, 3, and 6 weeks following treatment, mice were killed and samples of blood (0.5 mL) were collected using 1.5 mg/mL dipotassium EDTA as anticoagulant. The number of platelets per unit volume was determined by an automated analyzer (STK-S Coulter, Miami, FL).

As a further indicator of myelotoxicity, we determined in the same groups of mice the clonogenic potential of colony-forming unit (CFU)-A cells as described previously (35). CFU-A cells are transiently engrafting hemopoietic stem/progenitor cells whose daughters include all hemopoietic cell subpopulations, except lymphoid cells, and whose proliferative capacity is indicative of the status of the hemopoietic stem cell compartment in general (36).

Platelet and CFU-A colony counts of the various treatment groups were compared by Student's *t* test.

Statistical analysis. Statistical analysis was done as described above in the methods for each type of experiment. In pairwise comparisons of different treatments in the comet assay and *in vivo* therapy studies, *P*s are reported without adjustment for multiple comparisons. To ensure a type I error rate of <5% in the set of comparisons considered of primary importance (i.e., those among the three combination treatment schedules) while preserving reasonable power, differences with $P < 0.016$ were considered significant. In all other tests, $P < 0.05$ was considered significant.

Statistical analysis was done using GraphPad Prism version 3.0 (GraphPad Software, Inc., San Diego) and SPSS version 9 (SPSS, Inc., Chicago, IL).

Results

[^{131}I]*meta*-iodobenzylguanidine uptake by cell lines

Untransfected UVW/NAT cells showed negligible active accumulation of [^{131}I]MIBG. UVW/NAT cells containing the NAT cDNA had 30-fold higher [^{131}I]MIBG uptake (224,700 counts/min/10^6 cells) than the same cells treated with the specific monoamine uptake inhibitor, desmethylimipramine. The active uptake of [^{131}I]MIBG by UVW/NAT cells was approximately twice that of SK-N-BE(2c) cells (102,500 counts/min/10^6 cells), which exhibit endogenous *NAT* gene expression (19, 20).

Cytotoxic synergism between topotecan and [^{131}I]*meta*-iodobenzylguanidine

The effects of topotecan and [^{131}I]MIBG on SK-N-BE(2c) and UVW/NAT cells as single agents or in combination are shown in Fig. 1. From these survival curves (Fig. 1), median-effect plots were constructed (Fig. 2) to enable the calculation of various levels of toxicity. CIs were determined using the second equation. As the manner of the interaction between the agents was uncertain, the formula was solved in two different ways. CIs are presented graphically as solid lines (Fig. 2) or as broken lines (Fig. 2), corresponding to modes of action of the two agents that are similar or distinct, respectively. CI < 1, CI = 1, and CI > 1 indicate synergism, additivity, and antagonism, respectively.

SK-N-BE(2c) cells. Supra-additive kill of SK-N-BE(2c) cells after treatment with all combination schedules was exemplified by CIs < 1 at the IC_{10} and IC_{30}. For example, at the IC_{30}, CIs were 0.501 ± 0.117 or 0.566 ± 0.147 for schedule 1, 0.282 ± 0.037 or 0.302 ± 0.043 for schedule 2, and 0.260 ± 0.018 or 0.277 ± 0.020 for schedule 3. However, at levels of toxicity greater than IC_{50}, the combined treatments were less effective than the individual drugs and CIs > 1 were obtained at the IC_{70} and IC_{90}. At the IC_{50}, in schedule 1, the CI calculated assuming mutual exclusivity was slightly <1 (0.922 ± 0.027), whereas the CI calculated assuming independent modes of action of the two agents was slightly >1 (1.134 ± 0.039). Conversely, the effects of combination schedules 2 and 3 were synergistic at the IC_{50} regardless of the method of derivation of CI (schedule 2: 0.562 ± 0.015 or 0.641 ± 0.019; schedule 3: 0.507 ± 0.043 or 0.572 ± 0.054).

UVW/NAT cells. Unlike the response of SK-N-BE(2c) cells, synergistic interaction was achieved with all three schedules of administration when UVW/NAT cells were exposed to combinations of topotecan and [^{131}I]MIBG at doses that sterilized >50% of clonogens. For example, at the IC_{70}, CIs were 0.672 ± 0.108 or 0.785 ± 0.144 for schedule 1, 0.455 ± 0.057 or 0.507 ± 0.070 for schedule 2, and 0.615 ± 0.097 or 0.710 ± 0.126 for schedule 3. In contrast, at the IC_{10}, and IC_{30}, topotecan and [^{131}I]MIBG interacted antagonistically regardless of the scheduling of the agents.

For both SK-N-BE(2C) and UVW/NAT cells, at every level of toxicity analyzed, treatment with topotecan before exposure to [^{131}I]MIBG was less effective than administration of topotecan either after or simultaneously with [^{131}I]MIBG.

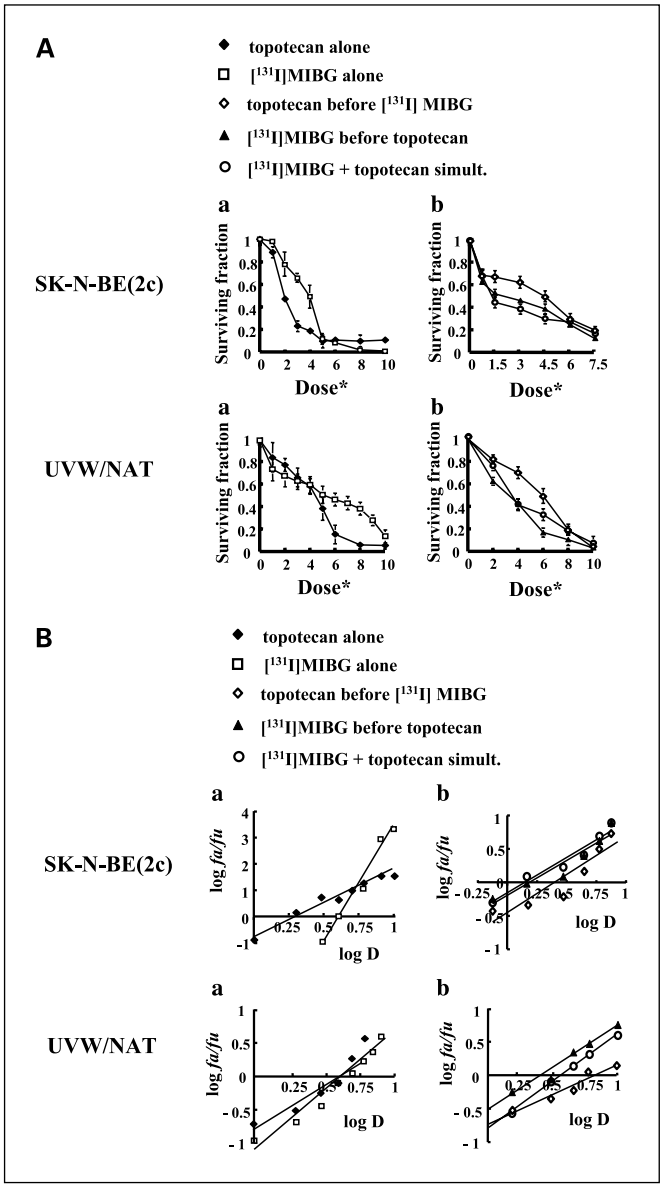

Fig. 1. *A,* clonogenic survival following the treatment of SK-N-BE(2c) and UVW/NAT cells with topotecan and [^{131}I]MIBG as single agents (*a*) or in combination (*b*). Asterisks, in the case of single-agent treatments, 1 dose unit = 27 μCi/mL [^{131}I]MIBG or 1 ng/mL topotecan. In combination treatments, SK-N-BE(2c) cells were treated with topotecan and [^{131}I]MIBG in a fixed 1:2 ratio. Therefore, 1 dose unit = 0.33 ng/mL topotecan + 18 μCi/mL [^{131}I]MIBG. UVW/NAT cells were treated with topotecan and [^{131}I]MIBG in a fixed 1:1 ratio. Therefore, 1 dose unit = 0.5 ng/mL topotecan + 13.5 μCi/mL [^{131}I]MIBG. *B,* median effect plots: SK-N-BE(2c) and UVW/NAT cells exposed to (*a*) single-agent treatment and (*b*) combination treatment. These plots were constructed from the data in (*A*) using the equation: $\log D = \log (D_m[f_a/f_u]^{1/m})$, where D is the dose, D_m is the IC_{50} dose, f_a is the fraction of cell affected, f_u is the unaffected fraction, and m is the coefficient of the sigmoidicity of the dose-effect curve. From the plots of log D against log f_a/f_u, the x-intercept (log IC_{50}) and slope m were calculated for each drug and for combinations by the method of least squares. These variables were then used to calculate the doses of the component agents and combinations required to produce various cytotoxicity levels according to the method of Chou and Talalay (27).

DNA damage

Figure 3 shows DNA fragmentation (expressed as tail moment) of SK-N-BE(2c) and UVW/NAT cells in response to treatment with topotecan or [^{131}I]MIBG alone or in combination. Assays were conducted either immediately or, to allow repair of DNA, 24 hours after treatment.

Following treatment of SK-N-BE(2c) cells with topotecan alone, [^{131}I]MIBG alone, or topotecan scheduled before [^{131}I]MIBG, significantly smaller comet sizes were observed when cells were assayed 24 hours after treatment rather than immediately after treatment ($P < 0.01$). In contrast, when topotecan was scheduled either after or simultaneously with [^{131}I]MIBG, no significant change in comet size was observed between 0- and 24-hour time points. This suggests that treatment schedules 2 and 3 have the capacity to impair the repair of DNA damage in SK-N-BE(2c) cells.

A similar repair response to the various treatments was noted in UVW/NAT cells. However, unlike SK-N-BE(2c), where significant reduction of comet size was dependent on scheduling, in UVW/NAT cells, a significant decrease in tail moment was observed in every treatment group 24 hours after treatment ($P < 0.01$). Nonetheless, in common with the response of SK-N-BE(2c) cells, the 24-hour levels of DNA damage manifested by UVW/NAT cells, which received treatment schedule 2 or 3, were significantly higher ($P < 0.01$) than those of untreated controls, cells exposed to single-agent therapy or combination schedule 1.

In vivo antitumor effects of [^{131}I]*meta*-iodobenzylguanidine and topotecan alone or combined

None of the animals in this study showed signs of distress. Figure 4 shows the effect, on the growth of two tumor xenografts, of the administration of topotecan or [^{131}I]MIBG either alone or in combination. Tumor growth times and cure rates are presented in Table 1.

SK-N-BE(2c) tumors. Overall differences in the effectiveness of the different treatments were highly significant (Kruskal-Wallis test, $P < 0.001$). The tumors of mice that received >i.p. injection of saline (controls) had a volume doubling time (T_2) of 10.4 ± 2.0 days (mean ± SD). Single treatments with topotecan or [^{131}I]MIBG significantly increased the T_2 to 19.7 ± 6.6 days ($P < 0.001$) or 22.8 ± 10.1 days ($P < 0.001$), respectively. No complete tumor regression resulted from treatment with NaCl solution or from either single-agent therapy. Combination treatment schedule 1 produced a greater tumor growth delay (T_2 = 26.3 ± 10.3 days) than either drug alone, but no tumor was cured and the increase in growth delay was not statistically significant. Administration of the two agents by schedule 2 produced a greater T_2 (34.3 ± 8.3 days) than schedule 1 and sterilized 1 of 12 tumors. This schedule was significantly more effective than either topotecan ($P < 0.001$) or [^{131}I]MIBG ($P < 0.01$) given alone. The most effective tumor control was obtained when the two agents were given simultaneously (schedule 3). The resulting T_2 (53.2 ± 7.5 days) and cure rate of 5 of 12 tumors (42%) represented significantly greater efficacy than the other two combination treatments ($P < 0.001$).

UVW/NAT tumors. Overall differences in the effectiveness of the different treatments were again highly significant (Kruskal-Wallis test, $P < 0.001$). In the NaCl solution–treated (control) group of mice, tumors reached a 10-fold increase in volume (T_{10}) in 18.6 ± 4.2 days (mean ± SD). None of the tumors in this control group spontaneously regressed. Tumors treated with topotecan alone had greater T_{10}s than the saline group (25.3 ± 6.1 days) and 2 (17%) tumors were cured. In mice that received [^{131}I]MIBG alone, a T_{10} of 31.9 ± 7.4 days and a cure

rate of 50% were achieved. Both single-agent treatments were significantly more effective than saline (topotecan, $P < 0.01$; [^{131}I]MIBG, $P < 0.001$).

The T_{10} (37.1 ± 12.8 days) corresponding to the delivery of [^{131}I]MIBG 24 hours after injection of topotecan (schedule 1) was greater than those of the groups that received single-agent treatment, and the cure rate was 50%, although this improvement was not statistically significant. When topotecan was given 24 hours after [^{131}I]MIBG (schedule 2) rather than in the reverse order (schedule 1), a further increase in T_{10} (49.7 ± 12.0 days) was observed and 67% of tumors were cured. This represented a significant improvement in efficacy relative to topotecan alone ($P < 0.001$) but not relative to [^{131}I]MIBG alone or to schedule 1.

A more dramatic effect was noted when the two agents were given simultaneously (schedule 3). Concomitant delivery of [^{131}I]MIBG and topotecan cured all UVW/NAT tumors. This constituted a significant improvement in efficacy relative to combination schedule 1 as well as both single-agent treatments ($P \leq 0.014$) although not relative to schedule 2.

Assessment of marrow toxicity of [^{131}I]*meta*-iodobenzylguanidine and topotecan alone or combined

In mice hosting SK-N-BE(2c) tumors and treated with 486 μCi [^{131}I]MIBG, there was a significant decline in CFUs and thrombocytes at 1 week after [^{131}I]MIBG treatment with or without topotecan ($P < 0.01$). No difference in bone marrow toxicity was observed between topotecan and non-topotecan-treated animals. Recovery to normal values was observed at 2 weeks. In mice bearing UVW/NAT tumors and treated with 270 μCi [^{131}I]MIBG, no evidence of thrombocytopenia was observed up to 6 weeks after treatment with any of the three combination schedules. There was no significant difference in platelet count between any of the treatment groups. Likewise, the clonogenic potential of pluripotent stem cells was not adversely affected by any of the treatment regimens. Although CFU-A colony counts were, in most NaCl solution–treated animals, higher than those of the combination treatment groups, these differences did not reach statistical significance (data supplied but not shown).

Discussion

This study shows that the topoisomerase I inhibitor topotecan potentiated the therapeutic index of [^{131}I]MIBG targeted radiotherapy of tumors expressing the NAT in two nude mouse xenograft models. Several observations were made: (*a*) with respect to growth delay or cure of experimental tumors, simultaneous treatment with [^{131}I]MIBG and topotecan (schedule 3) was more effective than administration of topotecan 24 hours before [^{131}I]MIBG (schedule 1) or after [^{131}I]MIBG (schedule 2); (*b*) combination treatments caused only transient myelotoxicity (according to platelet production and CFU-A colony-forming capacity); (*c*) combination schedules 2 and 3 were more effective than schedule 1 with respect to clonogenic sterilization; and (*d*) repair of DNA damage was less efficient following treatment schedule 2 or 3 rather than schedule 1.

The two cell lines used in this study had similar radiosensitivities (37, 38) but differed with respect to uptake of [^{131}I]MIBG. SK-N-BE(2c) neuroblastoma cells endogenously express NAT, whereas UVW/NAT cells were transfected with the *NAT* gene. A plateau of toxicity was observed for both cell lines on exposure to topotecan alone, consistent with S-phase specificity of topoisomerase I inhibitors (15, 16, 39).

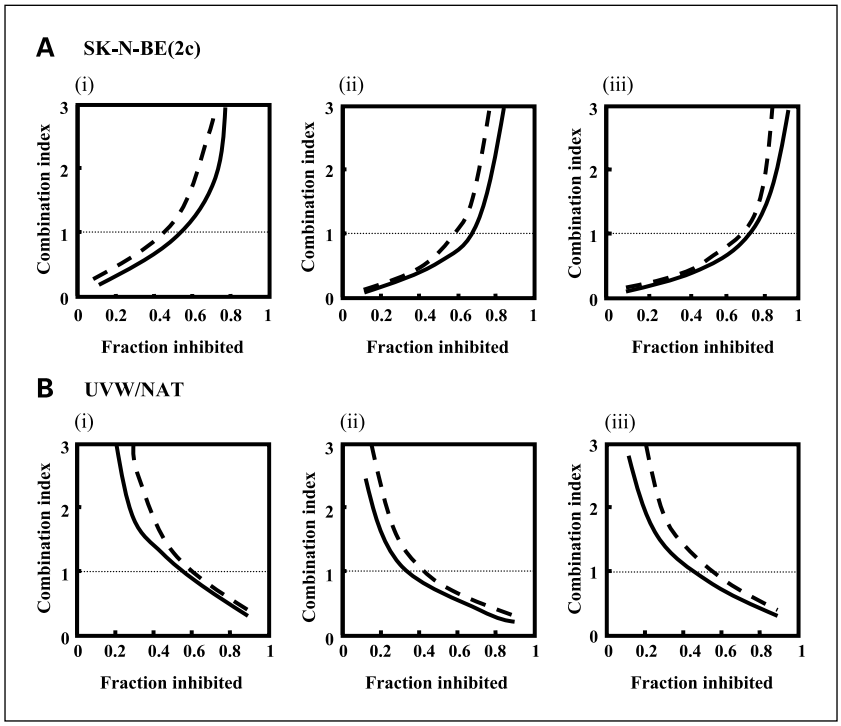

Fig. 2. CI versus cytotoxicity: interaction between topotecan and [^{131}I]MIBG in (*A*) SK-N-BE (2c) and (*B*) UVW/NAT cells. Topetecan was administered (*i*) before, (*ii*) after, or (*iii*) simultaneously with [^{131}I] MIBG. CIs are presented graphically as solid lines or as broken lines corresponding to modes of action of the two agents that are similar or distinct, respectively. CI < 1, CI = 1, and CI > 1 indicate synergism, additivity, and antagonism, respectively.

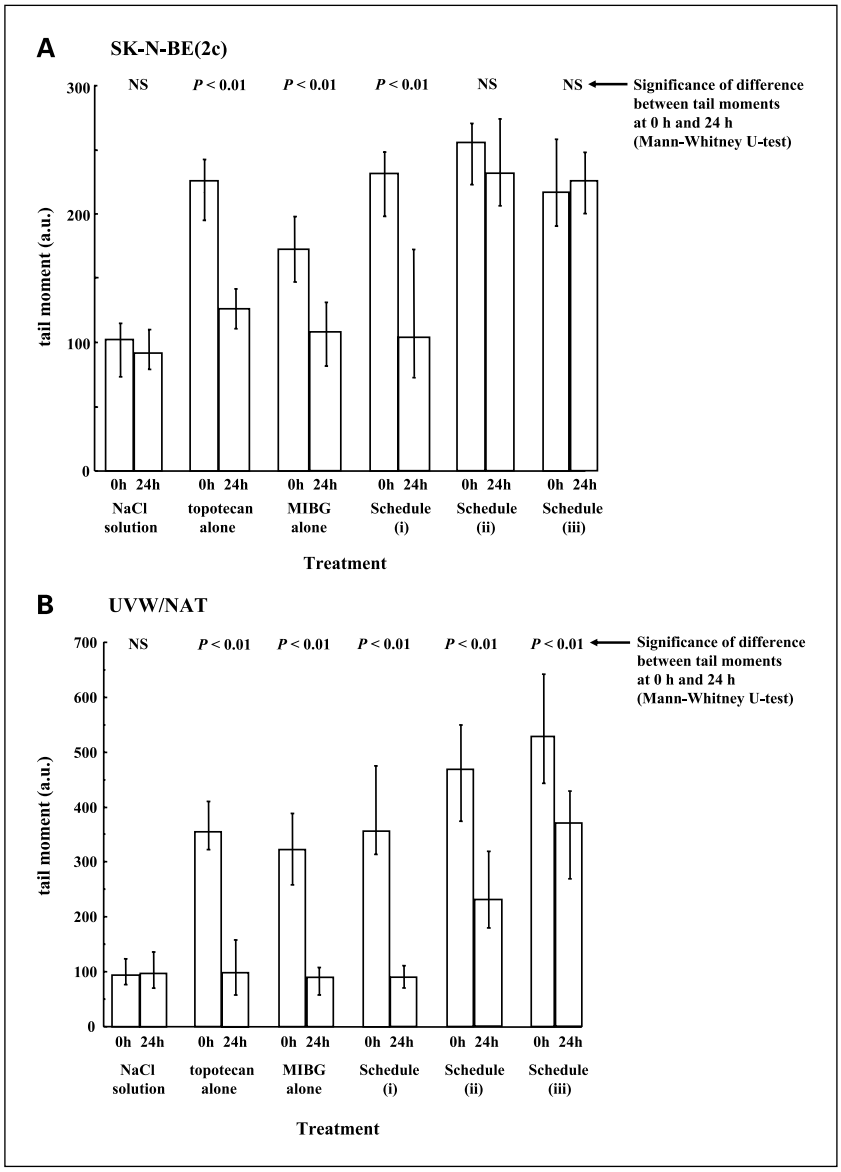

Fig. 3. DNA fragmentation after treatment of (*A*) SK-N-BE(2c) and (*B*) UVW/NAT cells with various combinations of [^{131}I]MIBG and topotecan. Median comet tail moments (and 95% confidence intervals) were determined either immediately or 24 hours after treatment (to allow for repair of DNA) with topotecan alone, [^{131}I]MIBG alone, or combinations of the two agents.

However, SK-N-BE(2c) cells exhibited greater sensitivity than UVW/NAT cells to topotecan at low doses of the drug, whereas at higher concentrations of topotecan a smaller proportion of UVW/NAT cells, compared with SK-N-BE(2c) cells, were resistant to treatment. Moreover, based on percentage decrease in comet tail moment 1 day after cytotoxic treatment, UVW/NAT cells exhibited more efficient DNA damage repair than SK-N-BE(2c) cells. These discrepancies between the response of SK-N-BE(2c) and UVW/NAT cells to experimental therapy may underlie their divergent responses to topotecan/[^{131}I]MIBG combination treatment. Supra-additive kill of SK-N-BE(2c) cells was obtained at low levels of toxicity. However, at higher toxicity levels, an antagonistic interaction was observed. This may be due in part to the higher proportion of non-S-phase SK-N-BE(2c) cells that are insensitive to topotecan. Combined topotecan and [^{131}I]MIBG treatment of UVW/NAT cells resulted in infra-additive kill at low levels of toxicity, perhaps resulting from resistance to low-dose topotecan and more efficient DNA repair. Conversely, higher doses of the two drugs (>IC_{50}) resulted in supra-additive sterilization of clonogens. Although synergism was dependent on the level of toxicity induced by the drug combinations, delivery schedules 2 and 3 were superior to schedule 1 at all intensities of clonogenic cell kill. This order of effectiveness of the sequence of treatments was reflected in their generation of long-term DNA damage, suggesting that substantial inhibition of repair of radiation-induced disruption of DNA may be affected by delivery of topotecan at the same time or after [^{131}I]MIBG.

Both [^{131}I]MIBG and topotecan produced significant inhibition of the growth of SK-N-BE(2c) and UVW/NAT tumors compared with controls. The administration of topotecan followed 24 hours later by [^{131}I]MIBG was more effective than single-agent treatments, but the improvement was not statistically significant. Scheduling of [^{131}I]MIBG before topotecan resulted in greater inhibition of the growth of SK-N-BE(2c) and UVW/NAT tumors than administration of

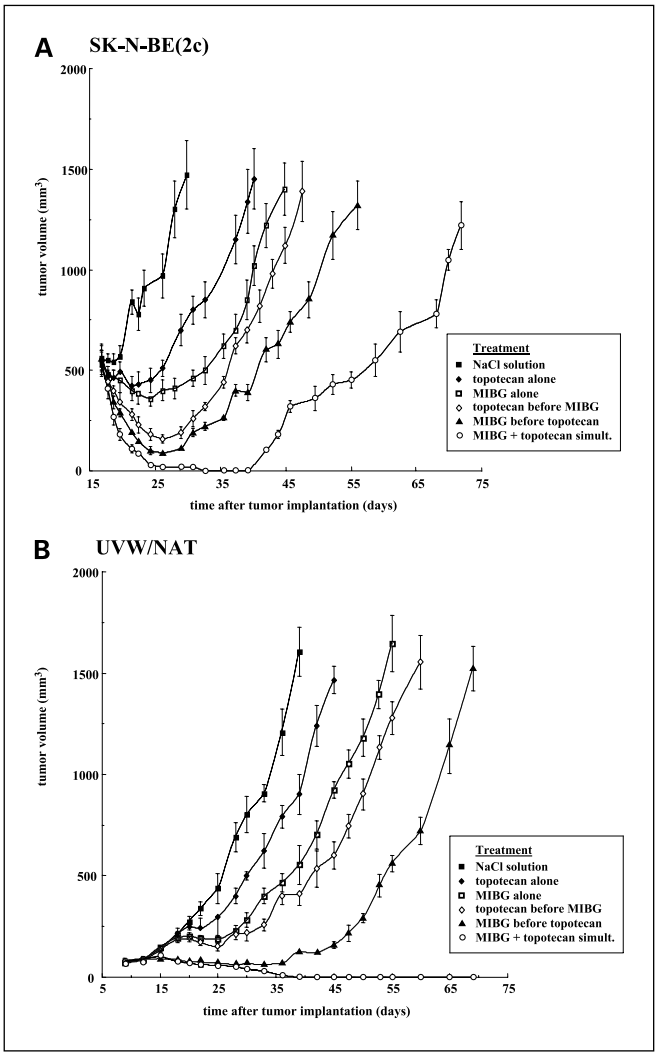

Fig. 4. Effect of combinations of [^{131}I]MIBG and topotecan on the growth of (*A*) SK-N-BE(2c) and (*B*) UVW/NAT tumors in nude mice. Each treatment group consisted of 12 animals. Points, means; bars, SDs.

the drugs in the reverse order, although this also did not reach statistical significance. This result, together with the observed inefficiency of DNA repair in response to combination schedule 2 or 3, suggests that any enhancement of tumor uptake of radiopharmaceutical by prior exposure to topotecan is less important to the outcome of therapy than the reduction in the efficiency of DNA damage repair in tumors exposed to topotecan. However, we cannot discount the possibility that targeted radiation may also have induced a delay in the progress of tumor cells through S phase, resulting in enhanced toxicity of topotecan. Striking tumor inhibition and tumor regression were produced by the simultaneous delivery of [^{131}I]MIBG and topotecan. This schedule sterilized 42% of SK-N-BE(2c) tumors and 100% of UVW/NAT tumors. The higher cure rate of the latter tumors is probably a reflection of their smaller size at the time of initiation of treatment.

The dose-limiting tissue in neuroblastoma patients treated with [^{131}I]MIBG is bone marrow, and toxicity is manifested by prolonged thrombocytopenia (40). Topotecan is also myelotoxic (41). Encouragingly, topotecan did not increase the concentration of activity in the bone marrow of experimental animals in the present study (data not shown). Therapeutically effective combination treatments induced, in one group of mice, a diminution of platelet numbers and decrease in the clonogenic capacity of CFU-A transiently engrafting stem cells. However, these indices of marrow integrity returned to normal levels 2 weeks after treatment. Hemopoietic stem cell rescue has been employed to minimize myelotoxic side effects of [^{131}I]MIBG therapy and this is feasible with acceptable toxicity (42).

Previous studies using *in vitro* and *in vivo* models have provided evidence for topotecan's enhancement of the effectiveness of external beam radiotherapy (17, 43) and radioimmunotherapy (44). Synergism may be due to diminished capacity for repair of radiation-induced DNA lesions (45) or to the accumulation of cells in S phase (resulting from radiation insult) where they are especially sensitive to topotecan (15). More recently, encouraging results have been obtained using topotecan combined with radiotherapy for patients with rhabdomyosarcoma (46), lung cancer (47), medulloblastoma, and supratentorial primitive neuroectodermal tumors (48). If the synergy shown in our model systems between topotecan and [^{131}I]MIBG can be replicated in patients with neuroblastoma, there is potential for real therapeutic gain. Following the lead of the present investigation, clinical studies of topotecan given simultaneously

Table 1. Tumor cure and delayed tumor growth resulting from the administration of topotecan or [^{131}I]MIBG alone or in combination

SK-N-BE(2c) tumors		
Treatment	**T_2 (d)**	**Cure rate (%)**
NaCl solution	10.4 (2.0)	0
Topotecan alone	19.7 (6.6)	0
[^{131}I]MIBG alone	22.8 (10.1)	0
Combination schedule 1	26.3 (10.3)	0
Combination schedule 2	34.3 (8.3)	8
Combination schedule 3	53.2 (7.5)	42
UVW/NAT tumors		
Treatment	**T_{10} (d)**	**Cure rate (%)**
NaCl solution	18.6 (4.2)	0
Topotecan alone	25.3 (6.1)	17
[^{131}I]MIBG alone	31.9 (7.4)	50
Combination schedule 1	37.1 (12.8)	50
Combination schedule 2	49.7 (12.0)	67
Combination schedule 3	71.0	100

NOTE: Cure rates are expressed as percentages of the 12 animals in each treatment group. T_2s are the mean (SD) number of days required for a doubling of volume of SK-N-BE(2c) tumors. T_{10}s are the mean (SD) number of days required for a 10-fold increase in volume of UVW/NAT tumors. All tumors treated simultaneously with topotecan and [^{131}I]MIBG failed to regrow. These were assigned a T_{10} of 71, corresponding to the day of termination of the experiment. All other T_2 and T_{10} take into account only those tumors that regrew.

with and subsequently to [^{131}I]MIBG therapy have commenced (49).

In summary, our results indicate good prospects for combined topotecan and [^{131}I]MIBG therapy of neuroectodermal tumors. Elucidation of the basis for the positive interaction between these treatments may be provided by analysis of cell cycle redistribution induced by single and amalgamated treatments. Further refinements to this combination, such as fractionated delivery of the two agents and multiple drug interactions, are worthy of investigation.

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