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Inhibition of the Human Apurinic/Apyrimidinic Endonuclease (Ape1) Repair Activity and Sensitization of Breast Cancer Cells to DNA Alkylating Agents with Lucanthone

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Abstract. *Cells repair DNA damage via four main mechanisms, however, damage induced by alkylators and oxidative damage is predominantly repaired by the DNA base excision repair (BER) pathway. The AP endonuclease, APE1, is one of the main enzymes in the BER pathway. It is abundant in human cells and accounts for nearly all of the abasic site cleavage activity observed in cellular extracts. APE1 expression is elevated in a variety of cancers and a high APE1 expression has been associated with poor outcome to chemoradiotherapy. The small molecule lucanthone has been shown to enhance the killing ability of ionizing radiation in cells and preliminary evidence suggests that lucanthone may inhibit AP endonuclease. Given the role APE1 plays in repairing oxidative and ionizing radiation DNA damage, the reports of lucanthone as an ionizing radiation enhancer and the potential use of lucanthone as an AP endonuclease inhibitor, we examined whether lucanthone could inhibit APE1 endonuclease activity. We report that lucanthone inhibits the repair activity of APE1, but not its redox function or exonuclease activity on mismatched nucleotides. Lucanthone also appears to inhibit exonuclease III family members (APE1 and ExoIII), but not endonuclease IV AP endonucleases, nor bifunctional glycosylase/lyases such as endonuclease VIII or formamidopyrimidine-DNA glycosylase (Fpg). Furthermore, the addition of lucanthone inhibits APE1 repair activity from cellular extracts and enhances the cell killing effect of the laboratory alkylating agent methyl methanesulfonate (MMS) and the clinically relevant agent temozolomide (TMZ). Given these initial findings, it would be of interest to further develop lucanthone as an APE1 inhibitor through the use of structure-function studies as a means of enhancing the sensitization of tumors to chemotherapeutic agents.*

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Cells repair DNA damage *via* four main mechanisms: direct reversal, the DNA base excision repair pathway (BER), nucleotide excision repair and mismatch repair. BER effectively repairs the damage induced by alkylators and oxidative damage and, within the BER pathway, one of the main enzymes is the AP endonuclease, APE1 (1). APE1 is abundant in human cells and accounts for nearly all of the abasic site cleavage activity observed in cellular extracts (2). It is responsible for AP site recognition and hydrolyzation of the phosphodiester backbone immediately 5' to an AP site. APE1 can also hydrolyze 3'-blocking fragments from oxidized DNA producing normal 3'-hydroxyl nucleotide termini, thereby permitting DNA repair synthesis; it has activity as a 3'-repair diesterase (3-6) and recently has been shown to possess a 3' mismatch exonuclease activity (7, 8). Moreover, APE1 functions as a redox factor (*i.e.* redox effector factor-1 (ref-1)) maintaining transcription factors in an active reduced state (1, 9). Through its redox function, it participates in other crucial cellular processes including responses to oxidative stress, regulation of transcription factors, cell cycle control and apoptosis (1). Importantly, APE's redox and DNA repair functions appear to be regulated independently.

The functional importance of APE1 is demonstrated as either elevated or altered APE1 expression in a variety of cancers including breast, cervical, germ cell tumors, gliomas, rhabdomyosarcomas and non-small cell lung cancer (10-16). Furthermore, high APE1 expression has been associated with poor outcome and poor complete response rate to chemoradiotherapy, shorter relapse-free intervals, poorer

survival and high angiogenesis (1, 10-13, 17-21). Consequently, altering APE1's redox function or DNA repair activity may be an important target for chemotherapeutic agents.

The small molecule lucanthone is used extensively as an antischistosome agent with a proven safety record (22). It also enhances the killing effect of ionizing radiation in cells and inhibits the catalytic activity of topoisomerase II, essentially acting as a topoisomerase II "poison" (22, 23). This latter finding is of interest given the expanding data relating AP sites as topoisomerase II poisons (24-26). Additionally, there exists one preliminary account of lucanthone acting as an AP endonuclease inhibitor (22). Given the reports of lucanthone as an ionizing radiation enhancer and a potential AP endonuclease inhibitor, and the critical role APE1 has in DNA repair and transcription factor regulation, we undertook a series of studies to determine whether lucanthone was able to inhibit APE1 endonuclease activity and thereby enhance the cytotoxic effects of alkylating agents normally repaired by the BER pathway.

Materials and Methods

APE1 and other repair enzymes. Exonuclease III, endonuclease IV, endonuclease VIII and Fpg were purchased from Trevigen, Inc. (Gaithersburg, MD, USA). Human APE1 wild-type and mutant protein were overproduced using the vector pGEX-3X (Pharmacia Biotech, Uppsala, Sweden). They were purified as previously described (27, 28). Briefly, *E. coli* cells containing the pGEX-APE1 (or the C65A mutant) fusion constructs were grown overnight. Overnight cultures were diluted 1:10 in fresh, pre-warmed medium and grown for 1 h at 37˚C. Expression of the fusion proteins was induced by adding isopropyl-beta-d-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM and growing the cells for an additional 3 h at 37˚C. Cell lysates containing the recombinant proteins were loaded onto glutathione sepharose 4B columns and binding with glutathione sepharose overnight occurred on a rotator at 4˚C. The columns were washed with 20 column volumes of 10 mM Tris, pH 8.0. The APE1 wild-type and C65A mutant protein were cleaved on the columns from GST using Factor X protease, 2.5 units/ml at room temperature for 2 h. The cleaved proteins were eluted with 10 mM Tris, pH 8.0 and the samples collected were resolved using sodium dodecylsulfate polyacrylamide gel electrophoresis and visualized using Coomassie Blue staining to determine purity. All protein samples used for these studies were > 95% pure.

Repair enzyme assays. APE1, endonuclease IV and exonuclease III assay: the recombinant protein (APE1 and the C65A mutant, 0.8 ng, exonuclease III: 0.1 ng, endonuclease IV, 0.16 ng) or the cellular protein extract (0.4 μg) was incubated with different concentrations (0, 50, 100, 200, 400, 800 μM) of lucanthone in a total volume of 20 μl assay buffer (50 mM HEPES, 50 mM KCI, 10 mM $MgCI_2$, 1% BSA, 0.05% Triton X-100, pH 7.5) at 37˚C for 30 min. One μl 0.2 pmol 5'-hexachloro-fluorescein phosphoramidite (HEX)-labeled terahydrofuranyl (THF) oligo (the 26 bp oligonucleotide substrate containing a single THF reside in the middle, yielding a HEX-labeled 13 mer fragment upon repair) was added into the mixture and incubated for 15 min. The reactions were terminated by adding 20 μl formamide without dyes. Sample solutions (20 μl) were then applied to a 20% polyacrylamide gel containing 8 M urea in 1XTBE buffer at 300 volts for 40 min.

MDA-MD231 cells ($1X10^7$), in logarithmic growth phase, were harvested by trypsinization and suspended in serum-containing culture medium, then centrifuged at 1500g for 5 min. The cells were washed with 5-10 ml of PBS and recentrifuged, then resuspended in 0.5 ml PBS with 2 mM DTT and kept on ice. The cells were pulse-sonicated on ice at 45 Watts, three times for 15 sec each, then centrifuged at 14,000g at 4˚C for 10 min. The protein concentration was quantitated by measuring absorbance at 595 nm using the Bio-Rad Bradford Protein Assay. The protein extract was used in the APE1 endonuclease assay on the THF-oligonucleotide as described above.

Endonuclease VIII and Fpg assay: the recombinant proteins (endonuclease VIII, 1 ng and Fpg, 0.54 ng) were incubated with different concentrations (0, 50, 100, 200, 400, 800 μM) of lucanthone in a total volume of 20 μl assay buffer (25 mM HEPES-KOH, 0.5 mM DTT, 0.5 mM EDTA, 150 mM KCI, 1% glycerol, pH 7.8) at 37˚C for 30 min. One μl 0.2 pmol HEX-labeled 8oxoguanine (8oxoG) oligo (the 26 bp oligonucleotide substrate containing a single 8oxoG reside in the middle, yielding a HEX-labeled 13 mer fragment upon repair) was added into the mixture and incubated for 60 min. The reactions were terminated by adding 20 μl formamide without dyes. Electrophoresis was carried out as described above.

Cell culture. The human breast cancer cell line MDA-MB231 was obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in RPMI1640 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were maintained in a humidified incubator at 37˚C in an atmosphere of 5% CO_2 and 95% air.

Drug treatments and cell survival (colony forming) assays. Colony forming assays were used to compare the dose response of cells to methyl methanesulfonate (MMS) and temozolomide (TMZ) with and without lucanthone. Percent survival was calculated by normalizing the efficiency of colony formation at each drug dose to that of untreated control cells. MDA-MB231 cells, approximately 50% confluent and in logarithmic growth phase, were treated with different doses of MMS (0, 0.05, 0.10, 0.15 and 0.2 mM) or TMZ (0, 0.1, 0.5, 1.0 and 2.0 μg/ml) with or without 3 μM lucanthone (lucanthone was preincubated for 2 h before the addition of MMS or TMZ to the medium) for 1 h, harvested as described above, washed in PBS, counted and plated in triplicate at a density to give between 30 and 300 colonies per 10-cm dish. After 12 days, the plates were stained with methylene blue (0.5% methylene blue, 50% MeOH) and the colonies were scored.

Statistical analysis. P values for all cell survival data were generated using the one-way analysis of variance (ANOVA) test with Sigma Stat software (Jandel Scientific, Erkrath, Germany). All *p* values were two-sided, and *p* values <0.05 were considered as significant.

Results

We initially investigated whether the small molecule lucanthone can inhibit the endonuclease activity of APE1. Using the Hex-oligonucleotide assay (29), lucanthone

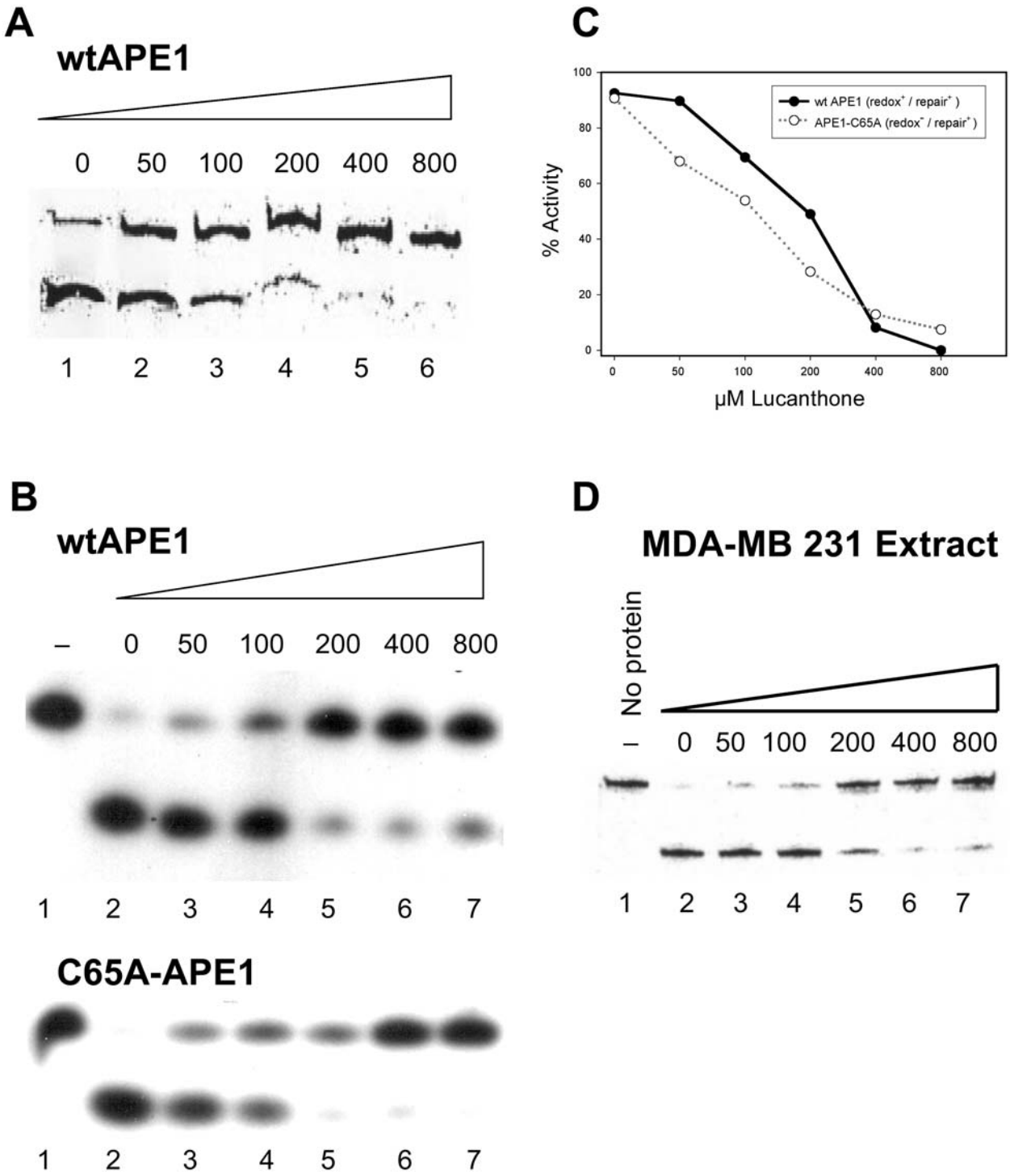

Figure 1. *Inhibition of endonuclease activity of APE1 and APE1 mutant C65A by lucanthone. Proteins used were: A) 800 pg recombinant wild-type APE1 (wtAPE1) protein preincubated with increasing amounts of lucanthone (µM) for 30 minutes and then assayed using the HEX-THF oligonucleotide substrate as described in Methods. Lane 1 is wtAPE1 without the addition of lucanthone as control. B) Either 800 pg wtAPE1 or APE1-C65A pretreated with lucanthone and assayed as in Panel A. The upper panel is wtAPE1 and the lower APE1-C65A mutant. C) Quantitation of the results shown in panel B. D) 400 ng of MDA-MB231 cellular protein extract preincubated with different concentrations of lucanthone for 30 minutes and assayed as described.*

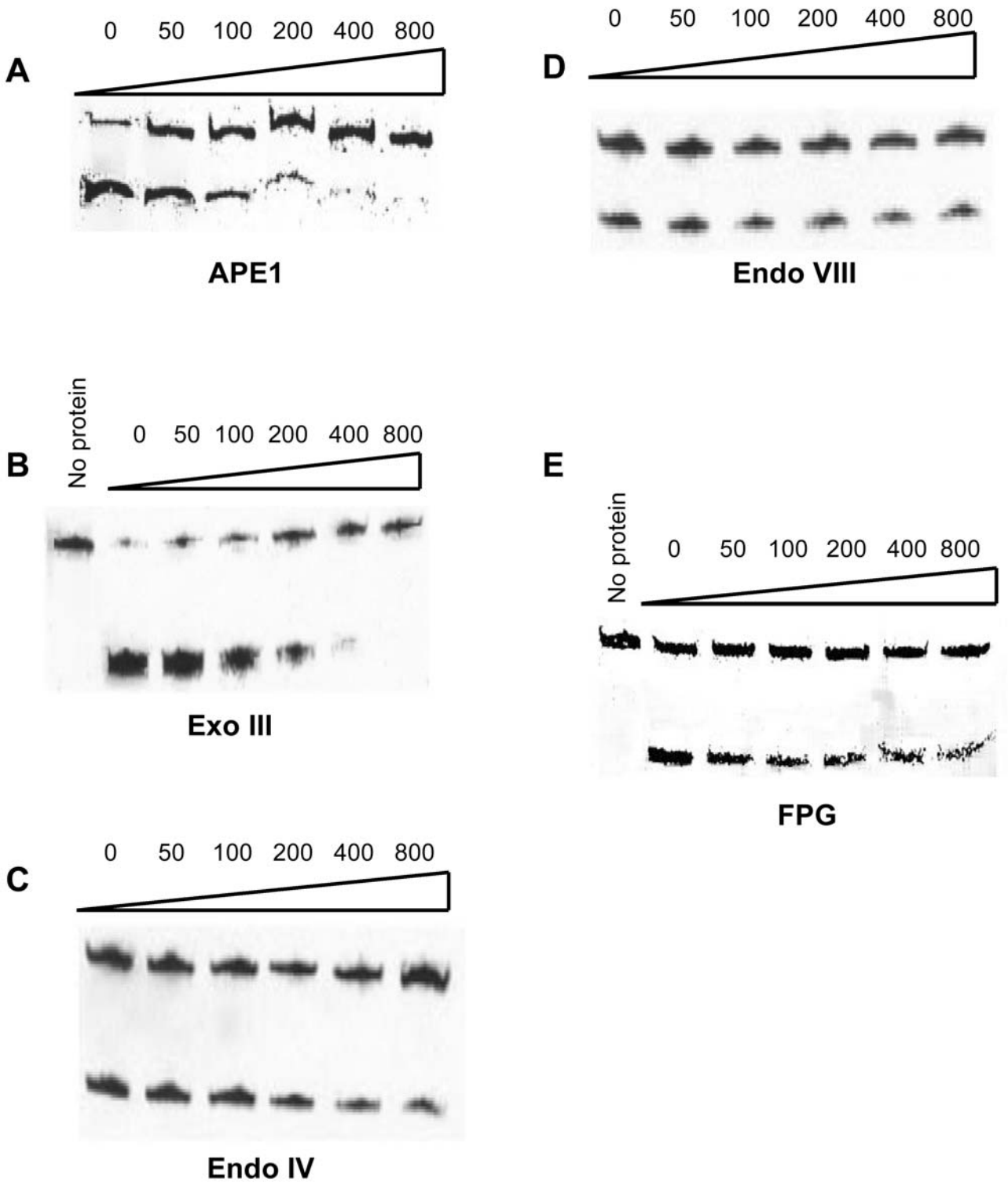

Figure 2. *Effect of lucanthone on activity of other DNA repair AP endonuclease or glycosylase/lyase enzymes. HEX-THF oligonucleotide assays were performed as described in Methods and Figure 1 using 0-800 μM amounts of lucanthone. A) 800 pg APE1, B) 100 pg exonuclease III, C) 160 pg endonuclease IV, D) 1000 pg endonuclease VIII and E) 540 pg Fpg.*

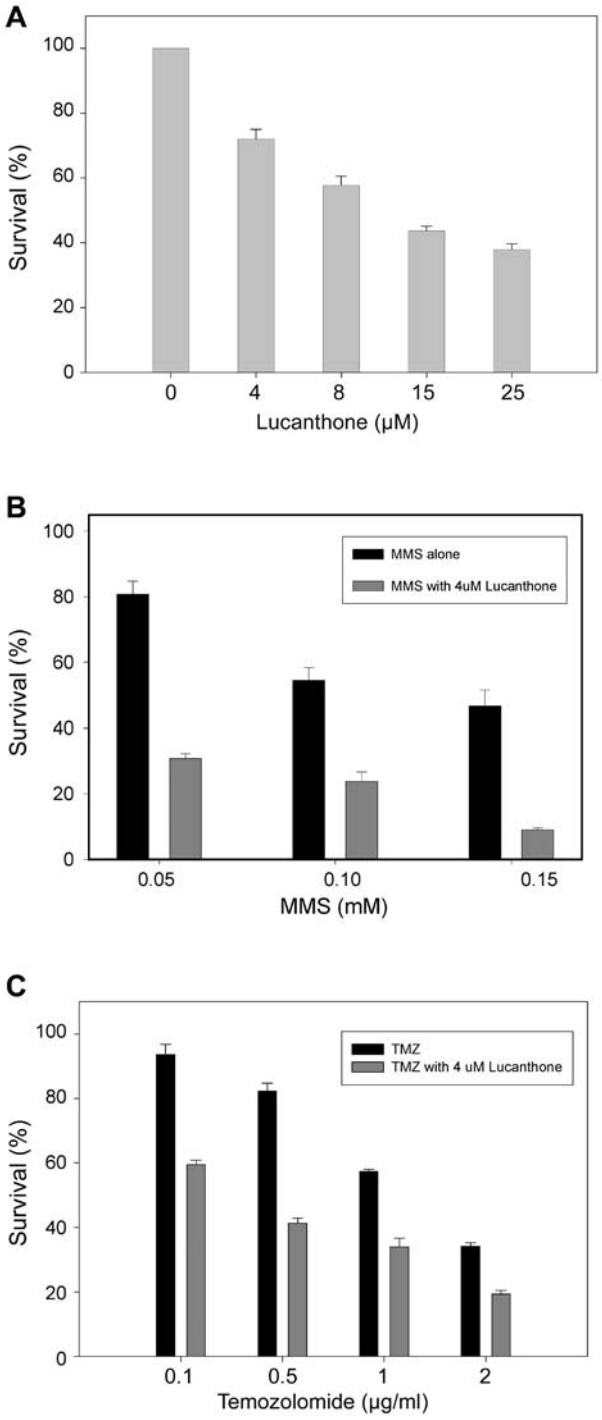

Figure 3. *Effect of lucanthone on cell survival . A) Breast cancer MDA-MB231 cells were treated with increasing doses of lucanthone for 1 hour and survival determined using the colony forming assay. B) Using a dose of lucanthone that caused minimal cell death (4 μM), MDA-MB231 cells were treated with either MMS with or without lucanthone (B) or TMZ with or without 4 μm lucanthone (C). Cell survival percentages were calculated based on the plating efficiency of the untreated cells. P values for all cell survival data were generated using one-way Analysis of variance (ANOVA) and at all doses the p values were <0.05.*

inhibited APE1 endonuclease activity starting at a 50 μM concentration using 800 ng of recombinant APE1 protein (Figure 1A). To determine whether this inhibition was specific, we repeated the AP-oligonucleotide assays using a mutant of APE1 that has been shown to be repair competent, but deficient in the redox function found in APE1 (1, 30), and has been shown to act as a dominant-negative in cell expression experiments (31-34). This mutant, APE1-C65A, has the cysteine at position 65 changed to an alanine. Lucanthone demonstrated similar patterns of endonuclease inhibition with both wild-type APE1 to the APE1-C65A mutant (Figure 1B and C). To confirm that this was not an artifact of using recombinant produced APE1, we performed AP endonuclease activity assays with a cellular extract from the human breast cancer cell line MDA-MB231. Again, the AP endonuclease activity, of which most, if not all, in the cellular extract is APE1, was inhibited by lucanthone at similar doses (Figure 1D)

Having established that lucanthone does inhibit both recombinant and cellular APE1, we were interested in determining whether this inhibition of AP endonuclease activity was specific for APE1 or just a non-specific inhibitory effect on any DNA repair endonuclease. There are two families of AP endonucleases; the exonuclease III family, of which APE1 is a member, and the endonuclease IV family which contains members such as *E. coli* endonuclease IV and yeast Apn1 (1, 35). Direct comparison of APE1 to *E. coli* exonuclease III and endonuclease IV demonstrated that lucanthone only inhibited exonuclease III at similar concentrations as observed with human APE1, but did not inhibit endonuclease IV (Figure 2A-C). We also tested the ability of lucanthone to inhibit representatives of the glycosylase/lyase family of repair enzymes. These enzymes remove damaged bases followed by sequential beta-elimination processing and are represented by the *E. coli* enzymes endonuclease VIII and Fpg. Both endonuclease VIII and Fpg act primarily on oxidized bases in DNA *via* the same mechanism, but have different substrate specificities (36). Lucanthone had no inhibitory effect on either of these enzymes (Figure 2D and E), even when the amount of enzyme used in these assays was much less than the amount of APE1 used; *i.e.*, the ratio of lucanthone to enzyme was greater than in the APE1/ExoIII experiments.

We also investigated whether lucanthone inhibited other functions ascribed to APE1: redox activity and the DNA exonuclease activity on mismatched deoxyribonucleotides at the 3' termini of nicked or gapped DNA molecules. There was no effect of lucanthone on APE1's redox function (data not shown), nor on its ability to act as an exonuclease (data not shown; personal communication, Dr. Chou, University of South Alabama, USA).

We next determined whether lucanthone alone or in combination with alkylating agents had any effect on cell survival, presumably through the inhibition of APE1 repair activity. First, we determined the dose of lucanthone that would give minimal cell toxicity effects on MDA-231 breast cancer cells. As demonstrated in Figure 3, lucanthone reduced the survival of MDA-231 by cells approximately 25% at the 4 μM dose (Figure 3A). We then used this dose of lucanthone with increasing does of either MMS (Figure 3B) or the clinically relevant alkylating agent TMZ (Figure 3C). With either drug, at all doses, we observed a clear enhancement of cell killing in the presence of lucanthone over drug alone. This enhancement was in the two-fold range (Figure 3).

Discussion

As a means to overcome tumor cell resistance and/or to sensitize tumor cells to currently used chemotherapeutic treatments, we have attempted to analyze the role DNA of repair inhibitors, particularly APE1 inhibitors, that may enhance the ability of alkylating agents to kill tumor cells. During the course of our investigations, we became interested in the small molecule lucanthone, which has previously been shown to enhance the killing ability of ionizing radiation in cells and has been shown to inhibit the catalytic activity of topoisomerase II, essentially acting as a topoisomerase II "poison" (22, 23). Additionally, one report suggested that lucanthone may act as an APE1 inhibitor (22). However, these data, although convincing, were preliminary in nature.

As demonstrated in this manuscript, lucanthone does appear to inhibit the repair activity of APE1, but not its redox function, nor its exonuclease activity on mismatched deoxyribonucleotides at the 3' termini of nicked or gapped DNA molecules, although these latter findings require further investigation. Furthermore, lucanthone appears to be specific for the exonuclease family of AP endonucleases (human and *E. coli*); it had no effect on the endonuclease IV or combination glycosylase/lyase repair enzyme families (endonuclease VIII, Fpg) (Figure 2). Given these data, it was hypothesized that lucanthone should sensitize human cancer cells to the effects of alkylating agents. This hypothesis is supported by previous studies showing that the reduction of APE1 with antisense methodology enhances cell killing to alkylating agents (37, 38).

As demonstrated in Figure 3, the combination of lucanthone and the laboratory alkylating agent MMS resulted in an enhancement of cell killing. Additionally, we see similar enhancement of cell killing using TMZ. TMZ is a clinical chemotherapeutic agent that has been effective in the treatment of astrocytoma, glioblastoma, brain metastases from other solid tumors and malignant melanoma (39). It has the distinction of being the first water-soluble and orally available chemotherapeutic capable of crossing the blood-brain barrier. Once administered, TMZ is non-enzymatically hydrolyzed in solution to the active compound 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC). This same active compound is also produced by cytochrome activation of the clinical prodrug dacarbazine. Because they share a common active form, the clinical use of TMZ has been guided by established uses for dacarbazine. By extension, TMZ will probably be effective in the treatment of lymphomas and all soft tissue sarcomas such as fibrosarcoma, rhabdomyosarcoma, leiomyosarcoma and neruoblastoma, which are recognized uses of dacarbazine not yet expanded to TMZ (40). TMZ, unlike dacarbazine, does not require enzymatic activation by liver enzymes for activity. This not only simplifies drug metabolism and interactions in patients, but allows for the use of TMZ in laboratory cell lines without requiring concomitant expression of cytochrome P450 enzymes.

In conclusion, we have demonstrated that the small molecule lucanthone effectively inhibits the repair activity of APE1. It also appears to inhibit exonuclease III family members (APE1 and ExoIII), but not the endonuclease IV AP endonucleases, or the bifunctional glycosylase/lyases, endonuclease VIII or Fpg. Furthermore, the addition of lucanthone inhibits APE1 repair activity from cellular extracts and enhances the cell killing effect of the laboratory alkylating agent MMS and the clinical agent TMZ.

Given these initial findings, it would be of interest to further develop this agent as an APE1 inhibitor through the use of structure-function studies to enhance the efficacy of lucanthone on repair inhibition and, ultimately, tumor sensitization to alkylating agents. Additionally, the combination of lucanthone and methoxyamine, a compound that covalently reacts with AP sites (41) and makes them refractory to repair by APE1 and DNA β-polymerase (42, 43), could be a potent combination for small molecule treatment of tumors undergoing alkylation and topoisomerase II drug treatments given the dual role of lucanthone.

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