Increased Death Receptor 5 Expression by Chemotherapeutic Agents in Human Gliomas Causes Synergistic Cytotoxicity with Tumor Necrosis Factor-related Apoptosis-inducing Ligand in Vitro and in Vivo

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Abstract

The intractability of malignant gliomas to multimodality treatments plays a large part in their extremely poor prognosis. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a novel member of the tumor necrosis factor (TNF) family that induces apoptosis preferentially in tumor cells through binding to its cognate death receptors, DR4 and DR5. Here we show that the DNA-damaging chemotherapeutic drugs, cis-diamminedichloroplatinum(II) (CDDP) and etoposide, elicited increased expression of DR5 in human glioma cells. Exposure of such cells in vitro to soluble human TRAIL in combination with CDDP or etoposide resulted in synergistic cell death that could be blocked by soluble TRAIL-neutralizing DR5-Fc or the caspase inhibitors, Z-Asp-CH2-DCB and CrmA. Moreover, systemic in vivo administration of TRAIL with CDDP synergistically suppressed both tumor formation and growth of established s.c. human glioblastoma xenografts in nude mice by inducing apoptosis without causing significant general toxicity. The combination treatment resulted in complete and durable remission in 29% of mice with the established s.c. xenografts and also significantly extended the survival of mice bearing intracerebral xenografts. These results provide preclinical proof-of-principle for a novel therapeutic strategy in which the death ligand, TRAIL, is safely combined with conventional DNA-damaging chemotherapy.

Introduction

Malignant gliomas, the most common primary brain tumors, remain largely incurable despite intensive multimodality treatment including surgical resection, irradiation, and chemotherapy. The 5-year survival rate of patients with glioblastoma multiforme, the most malignant form of glioma, remains at <10% (1). Besides the difficulty of complete surgical removal, resistance to chemotherapy and irradiation is also an important determinant for the successful treatment of malignant gliomas.

Apoptosis is a genetically controlled form of cell death that appears to be involved in tumor cell killing by most chemotherapeutic agents and by irradiation through disparate modes of action and cellular targets (2). Death ligands such as TNF and FasL (also called Apo1L/CD95L) interact with their cognate death domain-containing receptors, TNF receptor 1 and Fas (Apo1/CD95), respectively, thereby directly triggering suicide signal transduction pathways (3). Death signals evoked by the interaction of death ligands and their receptors may provide a new modality in glioma treatment because of their ability to directly induce apoptosis, thus bypassing cellular drug resistance. However, the therapeutic usefulness of FasL and TNF against cancer is limited by their acute toxicity after systemic administration, especially in the liver because of high expression of cognate receptors in hepatocytes, unless selectively targeted to tumor cells (4, 5).

TRAIL (also called Apo2L) is a new member of the TNF family and is capable of inducing rapid apoptosis in tumor cells of diverse origins but not in most normal cells in vitro (6–8). TRAIL can interact with two death receptors, DR4 (TRAIL-R1) (9) and DR5 (Killer/TRAIL-R2/TRICK2) (10–12), which contain cytoplasmic death domains, thereby triggering apoptotic signals. Such signals may be blocked by expression of the antagonistic decoy receptors, DcR1 (TRID/TRAIL-R3; Refs. 10, 11, and 13), DcR2 (TRUNDD/TRAIL-R4; Refs. 14–16), and osteoprotegerin (17), which can compete with DR4 or DR5 for binding to TRAIL. Both DR4 and DR5 transcripts are expressed to some extent in some cancer cells, but in the brain DR5 is expressed at low levels (7, 10, 11, 18). Unlike FasL or TNF, systemic administration of soluble human TRAIL has been shown to have little toxicity in mice or nonhuman primates (19, 20), suggesting its potential value for cancer therapy.

Because DNA damage has been shown to up-regulate DR5 transcription in some human cancer cells (21, 22), the possibility arose that conventional DNA-damaging chemotherapy might enhance the cytotoxicity of TRAIL through up-regulation of its receptor DR5. This might then lead to a synergistic activation by TRAIL-triggered apoptosis pathways. Here we tested this idea and show that the DNA-damaging agents CDDP and VP16, commonly used in human glioma treatment, caused up-regulation of DR5 transcripts in most glioma cells tested. Untreated cells were relatively resistant to TRAIL, and the drug-induced DR5 expression per se did not lead to apoptosis. However, treatment with soluble human TRAIL in combination with the DNA-damaging drugs caused a dramatic and synergistic cell death through TRAIL-receptor interaction and caspase activation. This combination treatment also significantly suppressed tumor formation as well as growth of established human glioblastoma xenografts in mice without generalized toxicity. These results suggest a novel and safe therapeutic strategy for the most aggressive type of glioma, glioblastoma, which combines conventional DNA-damaging chemotherapy with the apoptotic TRAIL ligand.

Materials and Methods

Chemicals. CDDP and VP16 were purchased from Sigma Chemical Co. (St. Louis, MO). BCNU was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD).
GLIOBLASTOMA TREATMENT WITH TRAIL AND CHEMOTHERAPY

Cells. The human glioblastoma cell lines used were described previously (23). The A1207 cell line was gift from Dr. S. Aaronson (Mount Sinai Medical Center, New York, NY). All cells were cultured as described (24).

Production and Purification of Human Recombinant Soluble FLAG-TRAIL. The plasmid encoding a soluble His-tagged FLAG-human TRAIL has been described (9). TRAIL was purified from plasmid-transformed BL21(DE3)pLysS cells by affinity chromatography on Ni2+-NTA agarose columns according to the manufacturer’s instructions (Qiagen, Santa Clarita, CA) and was then dialyzed against PBS containing 10% glycerol and stored at −80°C. To generate an endotoxin control for bacterial-derived TRAIL, lysates from mock BL21(DE3)pLysS cells were prepared and processed by the identical purification protocol. Soluble chimeric fusion proteins, DR5-Fc, and its Fc control were prepared as described (10).

RNA Analyses. Northern blot analysis was performed as described with modifications (24). A human DR5 cDNA fragment (583 bp) was generated by reverse transcription-PCR from total RNA of U87MG cells using primers 5'-CTGAAAGCGATCTGCTGAGTTG-3' (sense) and 5'-CAGGTTGCTCATTACCTCTTAG-3' (antisense). Hybridization was performed in ExpressHyb buffer (Clontech, Palo Alto, CA) at 68°C for 2 h. The RNA filter was washed in 0.5× SSC and 0.1% SDS at 58°C for 15 min and exposed to Kodak Biomax film at −80°C with an intensifying screen. RNase protection assays were carried out using the RiboQuant protocol according to the manufacturer’s instructions (PharMingen, San Diego, CA).

Cell Death Assays. Cytotoxicity was evaluated using the MTT survival assay as described (25). Briefly, cells were plated at 1 × 10⁴ cells/well in 96-well microtiter plates overnight. Cells were then treated with 200 μl fresh medium containing drugs, cultured for 24 h followed by an additional 4 h with 250 μg/ml MTT, and analyzed using a microplate reader ( Molecular Devices, Sunnyvale, CA). The effects of treatment are expressed as a percentage of growth inhibition using untreated cells as the uninhibited control. In vivo apoptosis assays were performed as described (24).

In Vivo Treatments. U87MG cells (2 × 10⁶ cells) were suspended in 0.1 ml PBS and injected s.c. into the right flank of female nude mice, 4–5 weeks of age, of BALB/c background (Simonsen’s Lab, Gilroy, CA). For the treatment of the established xenografts, the tumors were permitted to establish and grow for 13 days (tumor volume, <80 mm³). For intracerebral stereotactic inoculation, 5 × 10³ U87MG cells in 5 μl of PBS were inoculated into the right corpus striatum of the mouse brain as described (24). Either CDDP (3 mg/kg) or sterile normal saline was administered i.p. daily for 3 consecutive days per one course of treatment. Mice were also treated with either FLAG-TRAIL (250 μg) or the mock control lysate in the same volume twice a day with i.v. and i.p. injections on the same days as the CDDP treatment. The growth of tumors was measured as described (26). Systemic toxicity of the treatments was assessed by change in body weight and by organ inspection at autopsy. All treatment protocols were approved by the animal care and use committee of the University of California, San Diego.

Statistical Analysis. The data were analyzed for significance by Student’s t test, except for the in vivo survival assays, which used Cox-Mantel analysis. Synergism between TRAIL and DNA-damaging agents was assessed by the Fractional Inhibition Method as described (27).

Results

Treatment of Human Glioma Cells with DNA-damaging Agents Induces the DR5 Receptor for TRAIL. To explore the possibility that DNA-damaging treatment could augment TRAIL cytotoxicity by up-regulating its receptors, we first determined whether chemotherapeutic agents commonly used in glioma therapy could increase DR5 expression in human glioma cell lines. Northern blot analysis using a human DR5 cDNA as a probe showed that the basal level expression of ~4.4 kb DR5 transcripts increased significantly after treatment with the DNA-damaging agents CDDP or VP16 for 16 h at sublethal concentrations in most cell lines (U87MG, A1207, U178MG, LN229, U251MG, and LN428) and to a lesser extent (T98G) or very little (U373MG and LNZ308) in a few others (Fig. 1, A and B; Table 1). The induction of DR5 mRNA by these agents was dose, drug type, and time dependent, with induction by VP16 being apparent after 2 h, by 16 h of CDDP exposure, and with the alkylating agent, BCNU, having lesser effects (Fig. 1C).

We also determined the expression levels of the other TRAIL receptors and of endogenous TRAIL itself in the three most responsive cell lines, after DNA damage, using a multiple probe-RNase protection assay (Fig. 1D). DR5 levels increased upon treatment with CDDP and VP16 in U87MG, A1207, and U178MG cells, consistent with the Northern blot analysis. DR4 was also expressed and somewhat up-regulated in U87MG and A1207 cells but to a much lesser extent than DR5. Expression of the two TRAIL decoy receptors, DcR1 and DcR2, was only detectable at trace levels in U87MG cells, and TRAIL transcripts were undetectable in all cells.

Treatment with a Combination of Soluble Human TRAIL and DNA-damaging Agents Causes Synergistic in Vivo Cytotoxicity through Caspase Activation. Having determined that DR5 expression was enhanced upon exposure to DNA-damaging agents in a majority of human glioma cell lines tested, we next asked whether a combination of its ligand, TRAIL, with the drugs would enhance cytotoxicity. We measured the viability of glioma cells upon single exposures to a wide range of concentrations of CDDP, VP16, or BCNU. In each responder, the concentrations leading to growth inhibition were greater than those required for DR5 induction. This allowed the selection of doses that were sublethal but sufficient to induce DR5 expression, and these were then tested in combination with a low concentration (0.1 μg/ml) of TRAIL that had no effect itself on cell viability. A combination of these low doses of CDDP or VP16 and TRAIL induced substantial cell death in a synergistic manner in those glioma cells whose DR5 was up-regulated with DNA-damaging treatment. Evident changes in morphology were typical of apoptotic cell death (Fig. 2, A–L), and this was confirmed by DNA fragmentation detected by TUNEL assays (data not shown). The combination treatment-induced apoptosis was mediated through caspase activation because cleavage of PARP, a substrate of effector caspases, was elicited by the combination of TRAIL with CDDP or VP16 but not by any of the single treatments (data not shown). The synergistic cytotoxicity and the PARP cleavage were inhibited by the caspase inhibitors Z-Asp-CH₂-DCB (Bachem, Torrance, CA; Fig. 2, F and L) and CrmA (data not shown). In contrast, combination treatment, even at high doses of CDDP or VP16, did not result in enhancement of TRAIL cytotoxicity in U373MG and LNZ308 cells. DR5 expression of which was not affected by DNA damage treatment, and caused moderate killing of T98G cells, whose DR5 induction had been shown to be moderate. The doses that were effective in the combination treatment were >100-fold lower for TRAIL and mostly less than half for CDDP or VP16 than those required to kill the cells when administered alone (Fig. 3, A and B; Table 1).

The synergistic cytotoxicity was abolished by competition for TRAIL by DR5-Fc, a soluble portion of the extracellular ligand-binding domain of DR5 fused to the Fc region of human IgG (10), but not by Fc alone (Figs. 2 and 3, A and B). In contrast to CDDP and VP16, the combination of TRAIL and BCNU resulted in a less effective, synergistic cytotoxicity only in A1207 cells, and there was no synergy with U87MG and U178MG cells (Fig. 3C), concordant with the poor induction of DR5 caused by BCNU. BCNU has also been shown to inhibit caspase activation (28), which may also explain the lack of synergism between TRAIL and BCNU.

Treatment of Animals with a Combination of TRAIL and CDDP Suppresses Tumor Formation in Vivo. We next determined whether the synergistic cytotoxicity induced by the combination of TRAIL with DNA-damaging agents in vitro could be demonstrated in vivo. We first tested the effect of combination treatment on tumor formation in nude mice. Mice were inoculated s.c. with U87MG cells and also treated with TRAIL (500 μg/day) and CDDP (3 mg/kg).
After the third course of treatment as described in “Materials and Methods,” mice treated with the combination of TRAIL and CDDP had not developed tumors. In contrast, tumors in the other groups had already begun to grow, although treatment with TRAIL or CDDP alone showed some initial suppression of tumor growth (Fig. 4A). Tumor formation was significantly suppressed in the combination group (P = 0.007 against CDDP alone group). Two (50%) of the four mice in the combination treatment group had no tumors 3 months after tumor cell inoculation (Fig. 4C). There was no significant body weight loss or any obvious neurological sequelae in mice of any of the treatment groups and also in treated mice without tumors. Histological analysis of the livers, kidneys, and brains of mice obtained at the end of the first course of treatment showed no appreciable changes, except for minor and nonspecific single cell necrosis in the livers in all groups. After the third course of treatment, only minor infiltration of monocytes and granulocytes was observed in the liver obtained from one animal in the combination treatment group, but there were no obvious necroses in the livers from any of the treated animals (data not shown). We further examined effects of the treatments on normal human astrocytes. Neither the treatment with TRAIL alone (10 μg/ml) nor combination of TRAIL (0.1 μg/ml) and CDDP (10 μg/ml) or VP16 (16 μg/ml) caused cytotoxicity in these cells in vitro (data not shown).

**Treatment of Animals Carrying Established Tumor Xenografts with the Combination of TRAIL and CDDP Causes in Vivo Growth Suppression.** We next determined the effect of the combination treatment against established tumors. Mice bearing s.c. U87MG tumor xenografts were treated systemically with TRAIL and CDDP as described in “Materials and Methods.” The tumors carried by mice treated with the combination of TRAIL and CDDP

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**Fig. 1. Induction of DR5 mRNA expression by DNA-damaging agents in human glioma cell lines.**

Cells were treated with either CDDP (A), VP16 (B), or BCNU (C) at the indicated doses for 16 h and were harvested for preparation of total RNA. Fifteen μg of total RNA were size fractionated in a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and probed with a human DR5 cDNA (upper panels). Methylene blue staining of 28S rRNA demonstrates loading of RNA in each lane (lower panels). D, RNase protection assay showing expression and induction of TRAIL and its cognate receptors by DNA-damaging treatments in human glioma cell lines. Six μg total RNA were isolated from cells before and 16 h after CDDP or VP16 treatment and were subjected to RNase protection assay using the multiple-probe RiboQuant protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression is shown as a loading control.
regressed after the first course of treatment and remained smaller than the original tumors when the third course of the treatment was finished. In contrast, tumors in animals treated with the mock control or with either TRAIL or CDDP singly grew significantly faster than those treated with the combination (CDDP versus combination: \(P = 0.002\); Fig. 4, B and C). The suppression of tumor growth was dose dependent for both TRAIL and CDDP (data not shown). Long-term treatment resulted in durable remission for some tumors of the combination group. Among 17 tumors (data not shown). Long-term treatment resulted in durable remission for some tumors of the combination group. Among 17 tumors treated with the combination of TRAIL and CDDP, 5 tumors (29%) achieved complete remission, and 1 tumor was not growing >3 months after tumor inoculation (Fig. 4C). This frequency of regression caused by the combination treatment is significantly higher than that for hundreds of established U87MG xenografts we have observed over many years (<2%). Cultured tumor cells derived from the xenografts that eventually developed despite the combination treatment were equally sensitive to the combination treatment as the original cells were in vitro (data not shown), suggesting an inadequate drug concentration in tumors that did not regress. The combination treatment significantly induced apoptosis in tumor cells in the established xenografts after the first course of treatment, as demonstrated by TUNEL assays (\(P = 0.003\) versus vehicle control; Fig. 4D). Similar suppression of tumor growth was observed in three other independent experiments (data not shown).

We also examined the potency of the combination treatment for brain tumors using intracerebral xenograft models. Mice stereotactically inoculated in the brain with U87MG cells were treated with three courses of TRAIL and CDDP beginning from day 7 after inoculation. The combination treatment with TRAIL and CDDP significantly extended the survival of mice bearing intracerebral xenografts when compared with either vehicle, TRAIL, or CDDP treatment alone (\(P < 0.01\); Fig. 4E).

Table 1 DNA damage-induced DR5 up-regulation and synergistic cytotoxicity by combination treatment with TRAIL and genotoxic agents in human glioma cell lines

<table>
<thead>
<tr>
<th>Cell line (Cell line)</th>
<th>CDDP ((\mu g/ml))</th>
<th>VP16 ((\mu g/ml))</th>
<th>BCNU ((\mu M))</th>
<th>Synergistic cytotoxicity (^d)</th>
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<tr>
<td>Responders</td>
<td></td>
<td></td>
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<tr>
<td>U87MG</td>
<td>+ (4)</td>
<td>+ (16)</td>
<td>± (100)</td>
<td></td>
</tr>
<tr>
<td>A1207</td>
<td>+ (10)</td>
<td>+ (16)</td>
<td>+ (200)</td>
<td></td>
</tr>
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<td>U178MG</td>
<td>+ (4)</td>
<td>+ (16)</td>
<td>+ (100)</td>
<td></td>
</tr>
<tr>
<td>LN229</td>
<td>+ (10)</td>
<td>+ (16)</td>
<td>− (100)</td>
<td></td>
</tr>
<tr>
<td>U251MG</td>
<td>+ (4)</td>
<td>+ (8)</td>
<td>+ (200)</td>
<td>+</td>
</tr>
<tr>
<td>LN428</td>
<td>+ (10)</td>
<td>+ (64)</td>
<td>− (300)</td>
<td>+</td>
</tr>
<tr>
<td>Intermediate-responder</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T98G</td>
<td>± (10)</td>
<td>− (128)</td>
<td>− (200)</td>
<td>≤</td>
</tr>
<tr>
<td>Nonresponders</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U373MG</td>
<td>− (40)</td>
<td>− (128)</td>
<td>± (100)</td>
<td>−</td>
</tr>
<tr>
<td>LN229</td>
<td>− (40)</td>
<td>− (128)</td>
<td>− (100)</td>
<td>−</td>
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</table>

\(^a\) More than 20% increase in the percentage of growth inhibition than simple addition of the percentage of growth inhibition of the two drugs was evaluated as +, more than 10% increase as ±.

\(^b\) At the same concentrations used in the treatment for DR5 up-regulation. Single treatment with each of these drugs at the same concentrations was ineffective in all cell lines.

\(^c\) TRAIL (0.1 \(\mu g/ml\)) was used for all combination treatments. Single treatment with 10 \(\mu g/ml\) TRAIL was ineffective in all cell lines.

Fig. 2. Synergistic cytotoxicity in vitro by combination treatment with TRAIL and DNA-damaging agents. A, untreated control U87MG cells. Cells were treated with either TRAIL (0.1 \(\mu g/ml\), G), CDDP (4 \(\mu g/ml\), B), or VP16 (16 \(\mu g/ml\), H). Cells were treated with either TRAIL (0.1 \(\mu g/ml\)) plus CDDP (4 \(\mu g/ml\), C–F) or with TRAIL (0.1 \(\mu g/ml\)) plus VP16 (16 \(\mu g/ml\), I–L) in the absence (C and I) or presence of either DR5-Fc (D and J), Fc (E and K), or Z-Asp-CH \(_2\)-DCB (200 \(\mu M\), F and L). All treatments were performed for 24 h.
Discussion

Certain cytokines of the TNF family, including FasL and TNF, directly trigger apoptosis in tumor cells by engaging their cognate receptors, which contain cytoplasmic death domains (3). However, the use of FasL or TNF to treat tumors has been substantially hampered by their severe toxicity \textit{in vivo} (4, 5). In contrast, TRAIL, a new member of the TNF family, induces rapid cell death preferentially in some tumor cells \textit{in vitro} (6–8) and appears to have limited toxicity for mice (19) or monkeys (20). Because death signals originate from the death receptors, their up-regulation in tumor cells might be expected to enhance ligand-induced cytotoxicity. Here we show that treatment of human glioma cells with DNA-damaging agents CDDP or VP16 significantly and predominantly up-regulated DR5 transcripts, consistent with other studies (21, 22). Treatments with TRAIL plus each of these drugs could then effectively and synergistically kill glioma cells whose DR5 was up-regulated by the drugs through caspase activation and induction of apoptosis; yet treatments with each drug alone were ineffective. Up-regulation of DR5 and TRAIL-receptor interaction were essential in this effect because TRAIL-neutralizing DR5-Fc abolished cytotoxicity of the combination treatment. Synergism between TRAIL and doxorubicin treatment has been shown in breast cancer cells \textit{in vitro} (27). In our case, treatment of mice with systemic administration of TRAIL plus CDDP caused significant suppression of both tumor formation and growth of established human U87MG xenografts in a synergistic fashion, including complete remission, and unlike FasL or TNF, did not cause generalized toxicity.

The drug-induced DR5 up-regulation and subsequent enhancement of TRAIL-mediated cytotoxicity in glioma cells may correlate with their p53 status, because DR5 was up-regulated upon DNA-damaging treatments in all p53 wild-type glioma cells (U87MG, A1207, and U178MG), whereas it was not in p53-mutated U373MG and LNZ308 cells (29). In addition, adenovirus-mediated wild-type p53 expression has been shown to increase DR5 transcripts in human cancer cell lines with mutated p53 (22). However, we have been unable to show the direct involvement of wild-type p53 in this phenotype thus far using several approaches, including disruption of p53 function by introducing p53DD, a dominant-negative p53 (30), in p53-positive glioma cells, introducing wild-type p53 in p53-mutated glioma cells, and using syngeneic mouse cells with or without intact p53 alleles (data not shown). DR5 was also up-regulated in other p53-mutated glioma cell lines, U251MG, LN229, and LN428 (29), consistent with the suggestion that there are p53-independent pathways leading to its transcription levels of TRAIL receptors, including DR5 by the doxorubicin treatment (27).
This raises the possibility that our combination treatment approach could target both p53-wild type and -mutated glioma cells. Regulation of DR5 expression may also depend on the type of DNA damage, because the primary DNA lesions induced by the drugs used here are distinct, and the level of DR5 up-regulation varied among them. Perhaps certain DNA lesions preferentially initiate downstream pathways that activate the transcriptional machinery for DR5 expression. Identification and activation of the factor(s) responsible for direct DR5 transactivation would provide an alternative approach to augment TRAIL-mediated treatment of tumor cells.

Systemic administration of soluble TRAIL did not cause apparent neurological disorders or histopathological changes. Normal human astrocytes in culture were also resistant to the soluble TRAIL or the combination of TRAIL and DNA-damaging agents, consistent with the report by Ashkenazi et al. (20). The trace level expression of DR5 transcripts in normal brain tissue may also account for the absence of TRAIL-related toxicity in the brain (10, 11). Thus, the differences in DR5 expression between normal brain and glioma cells may provide a therapeutic window through which glioma cells could be selectively killed by TRAIL. Although further pharmacokinetic studies of soluble TRAIL in the central nervous system remain to be done, our results suggest a novel therapeutic strategy in which death ligands are combined with conventional DNA-damaging chemotherapy.

Acknowledgments

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References


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