

A Proapoptotic Peptide for the Treatment of Solid Tumors¹

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Abstract

We have designed a novel peptide, DP1, which is able to mediate significant induction of apoptosis in solid tumors by local injection. This peptide, comprised of a protein transduction domain (PTD), PTD-5, fused to an antimicrobial peptide, (KLAKLAK)₂, was able to trigger rapid apoptosis in a variety of cell lines *in vitro*, including MCA205 murine fibrosarcomas and human head and neck tumors. Furthermore, direct injection of DP1 into day 7 established MCA205 tumors in C57BL/6 mice resulted in the induction of tumor apoptosis and subsequent reduction in tumor volume. These results suggest that DP1 may be used clinically to treat accessible solid tumors or as an adjuvant therapy in conjunction with radiotherapy, standard chemotherapy, immunotherapy, or surgical debulking.

Introduction

The clinical treatment of a variety of solid tumors, including head and neck cancers, gliomas, and colorectal cancers, is still relatively ineffective (1, 2). Surgery remains the primary clinical approach for the treatment of most solid tumors; however, its utility is constrained by issues of accessibility and the need to minimize risk of damage to unaffected neighboring structures. Similarly, practical considerations limit the use radiotherapy, particularly when radiation exposure would exceed acceptable levels for normal tissue when treating large tumors (3). Cancer immunotherapy strategies face complex clinical challenges and have yet to demonstrate efficacy when challenged with advanced local disease (reviewed in Ref. 4). Finally, any conventional chemotherapy or gene therapy approach must surmount the challenges of appropriate biodistribution and uptake and ultimately mediate tumor cell death while minimizing side effects. Thus, a need exists to develop potential therapeutic approaches that can efficiently target tumors and serve as a stand-alone therapy or as an adjuvant to existing clinical treatment modalities.

Recently, we have identified a novel class of PTDs³ able to mediate rapid and efficient transduction of large, supramolecular protein complexes and chimeric in-line fusions in a receptor-independent manner. Such PTDs can facilitate rapid internalization of a variety of cargos both *in vitro* and *in vivo* in a wide variety of cell types (5). We have demonstrated that PTDs can be used for efficient delivery of protein complexes into solid tumors. The transduction properties of these PTDs make them suitable for the delivery of biologically active agents, including proteins, peptides, and DNA (6, 7). To determine whether the PTDs could be used for delivery of therapeutic agents to tumor by direct injection, we examined the antitumoral properties of

an antimicrobial peptide, (KLAKLAK)₂ (8), when fused to peptide transduction domain. If internalized into eukaryotic cells, antimicrobial peptides can cause mitochondrial disruption by triggering mitochondrial permeabilization and swelling, resulting in release of cytochrome *c* and induction of apoptosis. Murine fibrosarcomas (MCA205) and human head and neck tumors (22B and 4129) lines were used to test the anticancer activity of the PTD-antimicrobial peptide fusion, termed DP1, *in vitro* and *in vivo*. Here, we report that DP1 was efficient at triggering rapid tumor cell death in culture and inducing regression of established day 7 tumors, without apparent toxicity. These results suggest that DP1 could be used clinically to treat accessible tumors as an adjuvant therapy in conjunction with radiotherapy, standard chemotherapy, or surgical debulking to extend excision margins.

Materials and Methods

Design and Synthesis of the Proapoptotic Peptide DP1. The previously characterized PTD-5 transduction domain was used, because it was shown to efficiently deliver avidin-linked β -gal (Sigma Chemical Co.) conjugated to biotinylated PTD-5 to murine fibrosarcomas *in vitro* and *in vivo* (5). PTD-5 was coupled to the antimicrobial peptide “KLA” [KLAKLAKKLAKLAK; (KLAKLAK)₂], separated by a diglycine spacer, to generate the pro-apoptotic peptide, “DP1” (RRQRRTSKLMKRGGKLAKLAKKLAKLAK). PTD-5 and the KLA domain were also synthesized individually to serve as negative controls for activity. Peptides were synthesized and then purified by reversed-phase high-performance liquid chromatography to >90% purity on an acetonitrile/H₂O-trifluoroacetic acid gradient and confirmed by ion-spray mass spectrometry (Peptide Synthesis Facility, University of Pittsburgh). Lyophilized peptides were reconstituted in PBS for use *in vitro* and *in vivo*. Solubilized peptides were quantitated by ninhydrin chemistry, using l-norleucine (Sigma Chemical Co.) as an internal standard.

In Vitro Assay for Cell Viability. The MCA205 murine fibrosarcoma line and human head and neck tumor clinical isolates (22B and 4129) were cultured in DMEM supplemented with 10% FCS in 96-well plates. DP1, KLA, or PTD-5 was added to each well, and viability was monitored by MTT assay. Peptide concentrations ranging from 1 to 100 μ M were added to fresh serum-containing medium in each of the wells and incubated for 3 h at 37°C. After 3 h, 10 μ l of a 5 mg/ml stock solution of MTT (Sigma Chemical Co.) were added to the wells, and cells were further incubated at 37°C from 3 h to overnight. Subsequently, medium was aspirated, and the insoluble formazan crystals were solubilized in a solution of 10% SDS. Absorbance was taken at $\lambda = 570$ nm with background subtracted at $\lambda = 630$ nm. Each sample point was performed in triplicate.

Western for Caspase 3 Cleavage. MCA205 cells were plated in 60-mm plates and grown to confluency. PTD-5, KLA, or DP1 was added to fresh, serum-containing DMEM to 10, 25, or 50 μ M concentrations and incubated at 37°C for 30 min. An equivalent volume of PBS was added as a negative control in one plate. Cells were trypsinized briefly, and both cells and supernatants were collected and spun down at 1,500 rpm at 4°C. The resulting pellet was rinsed one time in PBS. Pellets were resuspended in a 3 \times volume in buffer A (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1.0 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride, and 1 mM DTT) and subjected to three rounds of freeze-thaw, followed by centrifugation at 2,000 rpm for 5 min and then 14,000 rpm for 20 min at 4°C to pellet cellular debris. The soluble cytosolic fraction was retained for analysis by Western. Total protein was normalized by Bradford protein assay (Bio-Rad) prior to loading on a 4–20% Tris-HCl gel and confirmed by Ponceau S staining of the transferred blot. The membrane

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³ The abbreviations used are: PTD, protein transduction domain; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; β -gal, β -galactosidase.

was probed with the mouse monoclonal caspase 3 antibody, CPP32 (BD Transduction Labs), and probed with a rabbit antimouse-horseradish peroxidase secondary antibody (Sigma Chemical Co.) and subjected to enhanced chemiluminescence.

In Vivo Administration of DP1. C57BL/6 mice bearing day 7 tumors on each flank (seeded with 1×10^5 MCA205 cells) received injections daily for 10 days with a 50- μ l volume of 1 mM DP1 or KLA or a Tris-buffered saline mock (TBS) into both tumors. Five mice were used in each group. Tumor volume was estimated by multiplying maximum length \times width (2). In addition, C57BL/6 mice with single, day 12 tumors were injected with 1 mM DP1, KLA, or saline for 11 days. Ten mice comprised each group. On the final day, the mice were injected with the appropriate saline or peptide solution and sacrificed 3 h after injection. Tumors were excised, paraffin embedded, sectioned, and stained for TUNEL and counterstained with methyl green or stained with H&E to reveal histological architecture.

Results

DP1 Inhibits the Viability of MCA205 and Head and Neck Tumors *in Vitro*. Previously, the 12-mer peptide sequence, PTD-5, was shown to mediate efficient protein transduction of both an avidin- β -gal complex coupled to biotinylated PTD-5 and a chimeric eGFP fusion protein. PTD-5- β -gal complexes efficiently transduced MCA205 murine fibrosarcomas both *in vitro* and *in vivo* (5). Distribution of β -gal staining in the injected tumors suggested that PTD-5 could be used to deliver proteins or other therapeutic agents locally to the injection site. On the basis of this data, a proapoptotic peptide, DP1, was constructed by synthesizing PTD-5 coupled to the (KLAK-LAK)₂ antimicrobial peptide (KLA), separated by a diglycine spacer.

The ability of DP1 to impair cell viability, in comparison with the PTD-5 or KLA peptide domains by themselves, was first examined *in vitro* in the MCA205 murine fibrosarcoma line using an MTT assay. The addition of DP1 directly to the cell culture medium mediated a potent reduction in MCA205 cell viability (Fig. 1a). Induction of apoptosis was rapid, with observable changes in cell morphology as early as 20 min after administration of DP1 to cells *in vitro*. In contrast, neither the mitochondrial disruption domain alone (KLA) nor the transduction domain alone (PTD-5) significantly affected MCA205 viability (Fig. 1a). DP1 also was able to confer a significant reduction in the viability of two human head and neck carcinoma cell lines, 4129 (Fig. 1b) and 22B (Fig. 1c), in contrast to the negligible response observed in KLA-treated cells.

Addition of DP1 to MCA205 Cells *in Vitro* Triggers Caspase 3 Activation. Previous work has suggested that internalized antimicrobial peptides lead to cell death by disrupting mitochondrial membranes and activating caspases (9). Cytochrome *c* release from damaged mitochondria induces Apaf-1 oligomerization, caspase 9 activation, and subsequent pro-caspase 3 to caspase 3 conversion that is responsible for many of the hallmarks of apoptosis (10). To determine whether DP1 activates the caspase 3-mediated apoptotic pathway, the disappearance of the inactive pro-caspase 3 from the cytosolic fraction of MCA205 cells incubated with DP1 was examined by Western analysis. MCA205 cells incubated in 50 μ M KLA or PTD-5 exhibited no discernable decrease in cytosolic pro-caspase 3 compared with a saline control (Fig. 2). However, treatment with DP1 resulted in cleavage of pro-caspase 3 in a dose-dependent manner, marked by the disappearance of the *M_r* 31,500 band. In addition, after 30 min of incubation in 50 μ M DP1, all treated cells displayed apoptotic morphology under phase contrast microscopy (data not shown). Pro-caspase 3 cleavage mediated by DP1 corresponds to its ability to impair cell viability by MTT at the same concentration (Fig. 1a). Taken together, these data indicate that caspase activation may be one pathway through which DP1 exerts its effects on cell viability.

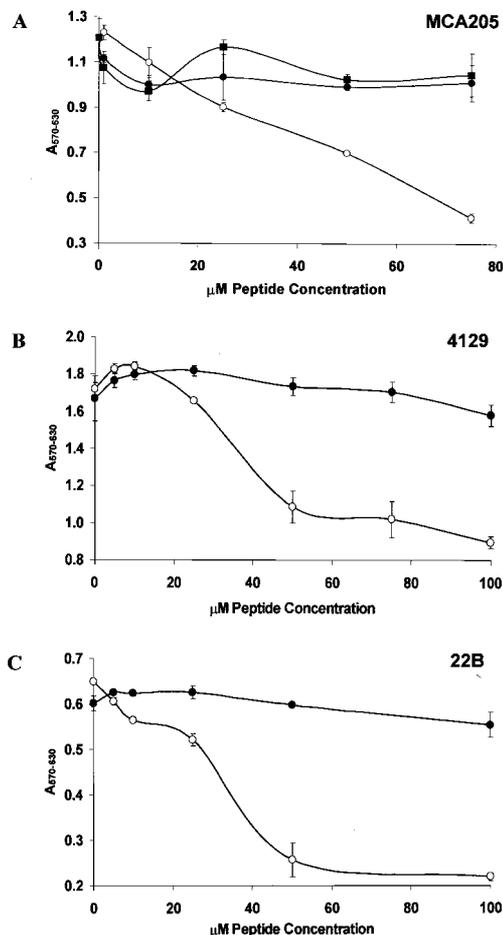


Fig. 1. *In vitro* impairment of cell viability by DP1 in MCA205, 22B, and 4129 cell lines. DP1 (\circ), KLA (\bullet), or PTD-5 (\blacksquare) was added to fresh medium in concentrations ranging from 1 to 100 μ M and incubated for 3 h at 37°C. Medium was removed, and MTT was added. Resulting formazan crystals were solubilized in 10% SDS. Results are shown for the MCA205 (A), 4129 (B), and 22B (C) cell lines. Means of experiments performed in triplicate for each group are plotted; bars, SD.

Direct DP1 Administration in Murine Fibrosarcomas Results in Tumor Regression.

To determine whether DP1 could be used to treat established murine solid tumors, we tested the efficacy of daily intratumoral DP1 administration on the growth of MCA205 fibrosarcomas *in vivo*. As shown in Fig. 3a, daily administration of a 50- μ l volume of 1 mM DP1 led to a striking reduction or complete halt in tumor growth in comparison to the KLA or TBS-treated controls. By the second day of treatment, $P < 0.05$ was reached between the DP1 *versus* the KLA or TBS-treated groups, as measured by a two-sample *t* test of unequal variances (Fig. 3a). As treatment continued, the differences between the DP1 and the KLA- or TBS-treated groups became more pronounced, with $P < 0.0003$ between the DP1 and KLA groups achieved by day 6. A plot of individual tumor sizes (Fig. 3b) demonstrates that by day 9 of treatment, no overlap in tumor sizes existed between the DP1-treated *versus* the KLA-treated cohorts. The appearance of tumors from the DP1- and KLA-treated groups 8 days after the initiation of treatment is shown in Fig. 3c. Notably, one of the DP1-treated tumors was completely eliminated after 1 week, with no growth apparent 9 days after the cessation of treatment. Resection of the treated area failed to reveal any evidence of residual tumor. In a separate experiment, 3 of 10 mice with single flank tumors treated with DP1 had undetectable tumors after 11 days of treatment (data not shown). Importantly, repeated administration of DP1 resulted in no apparent side effects in the treated mice.

To test whether an immune response against the tumors may have been triggered by apoptosis, DP1 treatment was halted at day 10. Tumors not completely eliminated by that time point grew back, in correlation with the cessation of DP1 administration. These data suggest that extensive induction of MCA205 tumor apoptosis by DP1 (Fig. 4) is ineffective at stimulating an antitumor immune response *in vivo*.

The Proapoptotic Peptide, DP1, Triggers Apoptosis *in Vivo*. To test whether apoptosis was indeed the mechanism of tumor killing *in vivo* and to study the effects of DP1 administration on tumor architecture, fibrosarcomas treated daily for 11 days with DP1, KLA, or saline (TBS) were injected with 50 μ l of 1 mM DP1, KLA, or TBS on the 11th day, and then mice were sacrificed 3 h later. TUNEL and H&E staining revealed that treatment with DP1 mediated significant apoptosis in MCA205 fibrosarcomas *in vivo* (Fig. 4). A broad zone of anuclear and acellular eosinophilic debris, ringed by a zone of tumor cells in the process of undergoing apoptosis, was evident in the DP1-treated but not KLA-treated tumors. The zones of killing were substantial; in some instances, an estimated 20% of total tumor volume stained TUNEL positive after DP1 administration. No TUNEL staining was observed in the KLA-treated tumors, indicating the requirement for coupling of (KLAKLAK)₂ to PTD-5 for induction of apoptosis. In addition, minimal lymphocytic infiltration of the treated tumors was observed, indicating that DP1 treatment does not incite a significant inflammatory response.

Discussion

We have demonstrated previously that a cationic peptide transduction domain, PTD-5, was very effective in delivery of a β -gal marker protein into established day 7 tumors. The percentage of tumor cells transduced with the PTD-5- β -gal complex was significantly greater than intratumoral injection of a high dose of an adenoviral vector carrying β -gal and with greater dissemination. This result suggested that PTDs could be highly effective for delivery of therapeutic agents by direct injection. In this study, we have examined the ability of PTD-5 to deliver a mitochondrial disruption domain to tumor cells in culture and *in vivo*. Our results demonstrate that a transduction domain-mitochondrial disruption domain fusion peptide, DP1, can mediate efficient killing of tumor cells both *in vitro* and *in vivo*. Induction of apoptosis was rapid and potent, with low concentrations of the DP1 peptide able to mediate cell death (LC₅₀ <50 μ M in MCA205 cells).

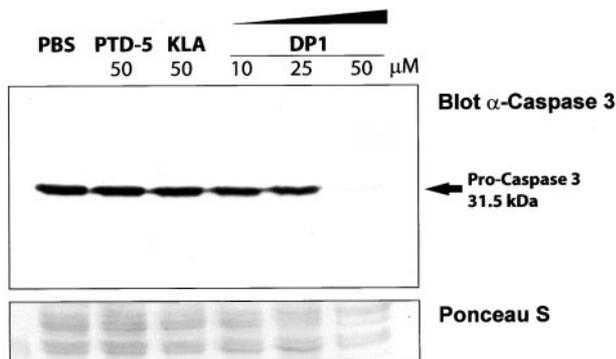


Fig. 2. DP1 triggers pro-caspase 3 cleavage in MCA205 cells. Cytosolic extracts from MCA205 cells treated for 30 min in serum-containing DMEM with PTD-5, KLA, DP1, or a saline control (PBS) were resolved by SDS-PAGE. Use of a mouse monoclonal caspase 3 antibody (CPP32) shows the disappearance of uncleaved, pro-caspase 3 from the cytosolic fractions, corresponding to caspase 3 activation. No significant difference in pro-caspase 3 processing among the saline, 50 μ M PTD-5, 50 μ M KLA, and 10 μ M DP1-treated cells was observed (Lanes 1–4). A slight increase in caspase 3 cleavage was observed in 25 μ M DP1-treated cells (Lane 5), with almost complete cleavage occurring at 50 μ M DP1 (Lane 6). Ponceau S staining (bottom panel) is shown to demonstrate total protein loading among lanes.

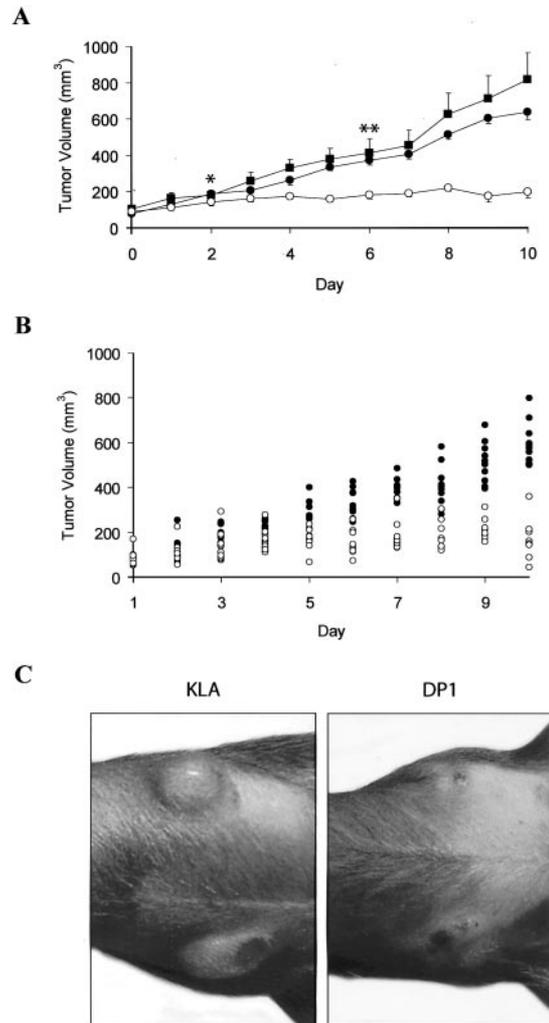


Fig. 3. *In vivo* tumor progression of DP1- and KLA-treated fibrosarcomas. Day 7 MCA205 tumors grown in C57BL/6 mice were injected daily for 10 days with 50 μ l of a 1 mM solution containing DP1 or KLA or TBS. Tumor volume (length \times width²) was measured each day. Results are shown (A), with the first day of peptide administration labeled as day 1. DP1-treated (\circ), KLA-treated (\bullet), and saline-treated (\blacksquare) tumors are plotted, with SDs shown as one-sided error bars around the mean of each data point. By the second day of treatment, after a single administration of peptide, $P < 0.05$ (*) was observed by a two-tailed Student's *t* test of the means between the DP1 and KLA groups. By day 6, $P < 0.0003$ (**) was reached between the groups. No statistically significant difference was observed between the TBS- and the KLA-treated tumors. Individual tumor sizes are shown in the scatter plot (B). \circ , DP1-treated tumors; \bullet , KLA-treated tumors. C, representative surface tumor morphology of the DP1- and KLA-treated mice at 8 days after the initiation of treatment.

Although this mechanism of killing is nonspecific, local administration of the peptide limits any observable regional or systemic toxicity, in contrast to many of the chemotherapeutic agents that have significant side-effect profiles. We have also attempted to specifically target head and neck tumors by substituting the PTD domain of DP-1 with the 12-mer peptide, HN-1, identified by biopanning against human head and neck squamous cancer cells (11). However, the resulting fusion peptide failed to display significant proapoptotic activity against a number of clinical head and neck tumor isolates that we tested, possibly because of either poor internalization or incorrect intracellular targeting.⁴

These data also demonstrate the modular nature of the transduction domain and the antimicrobial peptide acting as a mitochondrial disruption domain in DP1; each domain confers its properties upon the

⁴ Unpublished data.

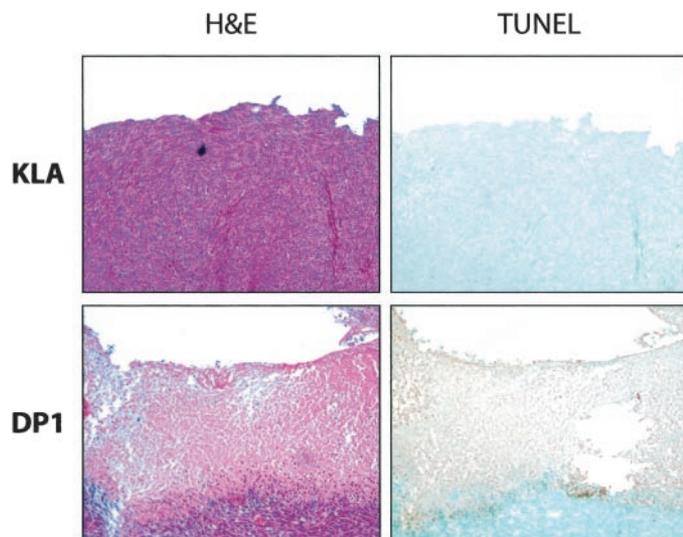


Fig. 4. DP1 but not KLA mediates apoptosis of MCA205 tumors *in vivo*. C57BL/6 mice with single flank, day 12 MCA205 tumors were injected daily for 11 days with 50 μ l of 1 mM DP1 or KLA. On day 11, mice were sacrificed 3 h after injection, and tumors were excised, paraffin embedded, sectioned, and stained. Representative H&E staining (left) and TUNEL (right) are shown for both groups.

coupled peptide product to generate a biologically active agent. We have exploited these characteristics to engineer second-generation peptides with enhanced proapoptotic activity by coupling them to antimicrobial peptides that are significantly more potent than (KLAK-LAK)₂.⁴ In addition, it points to the potential utility for using transduction domains coupled to other proteins, peptides, or compounds selective for neoplastic cells, much like Schwarze *et al.* (6) have demonstrated with an HIV-sensitive caspase 3.

It is possible that the DP1 fusion peptide can be used to facilitate an immune response against tumor antigens by triggering massive apoptosis within tumors (12). The correlation of resumed tumor growth with the cessation of treatment in our murine fibrosarcoma trial ruled out the possibility of a vigorous immune response against the tumor after 1.5 weeks of DP1 treatment. Nonetheless, the MCA205 fibrosarcoma line is particularly nonimmunogenic. It is possible that a more immunogenic tumor model might more readily prime the immune response after tumor cell apoptosis. Furthermore, it might be possible to augment the immune response against tumors by coadministration of cytokines, costimulatory molecules, or dendritic cells. *In vitro*, apoptotic bodies from B16 melanoma tumor cells treated with DP1 are readily taken up by murine dendritic cells, raising the

possibility that DP1 can be used for the *ex vivo* manipulation of immune responses.⁴

Proapoptotic peptides, such as DP1, may be used for the clinical treatment of a number of human solid tumors, including head and neck tumors, melanomas, and papillomas. Its potential utility may be primarily as an adjuvant in conjunction with preexisting treatment modalities, such as chemotherapy and/or radiotherapy. For example, advanced colorectal tumors can advance to the stage when they are so large that excision presents significant risk to the patient. In such scenarios, the application of such peptides can potentially assist in shrinking the tumor mass to a size that makes surgical excision a feasible option. In addition, DP1 may be used as an adjuvant therapy after surgical debulking of solid tumors to eliminate remaining neoplastic cells the perioperative region. Because DP1 is a peptide comprised of L amino acids, its proapoptotic effects are presumably limited by proteolytic degradation, as well as binding to serum components. As a bioablative agent, DP1 is expected to have low toxicity and high potency in comparison with many of the other cancer drugs that exist today.

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