A Novel Subclass of Thalidomide Analogue with Anti-Solid Tumor Activity in Which Caspase-dependent Apoptosis is Associated with Altered Expression of bcl-2 Family Proteins

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ABSTRACT

Thalidomide is clinically useful in a number of cancers. Antitumor activity may be related to a number of known properties, including anti-tumor necrosis factor (TNF)-α and T-cell costimulatory and antiangiogenic activities. However, it may also involve direct antitumor effects. A series of second generation thalidomide analogues have been separated into two distinct groups of compounds, each with enhanced therapeutic potential, i.e., SelCIDs, which are phosphodiesterase (PDE) type IV inhibitors, and IMiDs, which have unknown mechanism(s) of action. We report here our efforts to determine direct antitumor effects of thalidomide analogues and compounds from both groups. We found that one of the SelCID analogues (SelCID-3) was consistently effective at reducing tumor cell viability in a variety of solid tumor lines but had no effect on non-neoplastic cells. The antitumor activity was independent of known PDE4 inhibitory activity and did not involve cAMP elevation. Growth arrest was preceded by the early induction of G2/M cell cycle arrest, which led to caspase 3 mediated apoptosis. This was associated with increased expression of pro-apoptotic proteins and decreased expression of antiapoptotic bcl-2. Furthermore, extensive apoptosis in vivo was detected during SelCID-3-mediated inhibition of tumor growth in a murine xenotransplantation cancer model. Our results suggest that SelCID-3 represents a novel antitumor agent distinct from thalidomide and from previously characterized analogues with therapeutic potential against a range of solid tumors. This effect appears to be mediated via alterations in the expression of bcl-2 family proteins.

INTRODUCTION

Thalidomide has been shown to have clinical potential in a number of conditions in which there are limited therapeutic options, including cancer (1, 2). Clinical efficacy may be associated with a number of diverse properties attributed to thalidomide that include the inhibition of TNF-α synthesis (3), costimulation of T cells (4), and inhibition of angiogenesis (5).

The design and synthesis of thalidomide analogues is an ongoing research effort to obtain compounds with enhanced activity/toxicity profiles (6, 7). These analogues have been shown to segregate into at least two distinct classes. The SelCIDs (Selective Cytokine Inhibitory Drugs) consist of PDE4 inhibitors. The IMiDs (Immunomodulatory Drugs) are thought to be mechanistically similar to thalidomide (although with enhanced potency and therapeutic indices) and act via as yet unknown mechanism(s). Both groups of compounds are potent TNF-α inhibitors, although T-cell costimulatory activity is limited to the latter group (8).

Thalidomide and its IMiD analogues have recently been reported to possess antimyeloma activity in vitro (9). Furthermore, thalidomide and more recently the IMiD, CC-5013, have been used successfully to treat patients with multiple myeloma (10–13), and this has been extended to the treatment of patients with solid tumors (14–16). Thalidomide analogues are also being evaluated in vitro (8, 17–22), in vivo (23), and in the treatment of patients with end-stage cancer (24). However, there is little information concerning their direct effect on solid tumors. We, therefore, initiated a study to determine the relevance of direct antitumor activity and to identify potentially clinically effective compounds.

In this paper, we show that one of the SelCID analogues, SelCID-3, is effective at inducing cell cycle arrest in all eight of the lines tested. This activity is associated with alterations in the balance of pro- and anti-apoptotic bcl-2 family proteins and leads to caspase-dependent apoptosis. Furthermore, we show that SelCID-3 is an effective anti-tumor agent in a mouse xenograft model of cancer.

MATERIALS AND METHODS

Cell Lines and Culture. Tumor cell lines from three tumor types (colorectal: LoVo and SW620; pancreatic: BxPc-3 and T3M-4; prostate: PC-3 and DU-145) were originally obtained from American Type Culture Collection. Melanoma lines (MJJT-3 and SP-1) were derived from biopsy samples taken from patients attending the Oncology unit, St. George’s Hospital. All of the lines (except PC-3) were cultured in complete RPMI 1640 (Sigma, Poole, United Kingdom) + 10% FCS (Life Technologies, Inc., Paisley, United Kingdom), 1% penicillin/streptomycin, and 2 mM L-glutamine (all from Sigma). The prostate line PC-3 was grown in complete DMEM (Sigma). Non-neoplastic human foreskin fibroblast cell line HS 27 was obtained from ECACC (no. 94041901) and maintained in complete DME.

Thalidomide/Analogue and Other Reagents. Thalidomide and selected analogues (IMiD-1, SelCID-1, SelCID-2, and SelCID-3) were prepared at Celgene Corporation (Warren, NJ). Stock solutions (20 mg/ml) were freshly prepared in DMSO (Sigma) and diluted directly into cultured cells at up to 100 μg/ml. The caspase 3-specific inhibitor Z-DEVD-FMK was purchased from R&D Systems (Abingdon, United Kingdom). An inhibitor of protein kinase A-mediated signaling (14-22 Amide, cell permeable) was obtained from Calbiochem (Nottingham, United Kingdom).

MTS and XTT Cell Viability Assays. For the MTS assay, tumor cells were plated onto 96-well plates in complete medium and incubated at 37°C/5% CO2 for 24 h. Cells were then incubated with or without thalidomide or analogues (up to 0.5 μM final DMSO) for an additional 48 h when MTS/PMS (Promega, Madison) was added. After 1–2 h, A490 nm was measured.

The XTT Cell Proliferation Assay was carried out on the SW620 line in complete RPMI medium. The cells were pretreated with or without SelCID-3 at 37°C/5% CO2 for 1 h. The cells were then stimulated with or without forskolin (10 μM, 0.2% final DMSO) for 48 h. Cell viability was tested using Cell Proliferation Kit II (XTT; Roche Diagnostics, Mannheim, Germany). XTT labeling mixture was added to the cells and the A490 nm was measured after 2 h.

cAMP Accumulation Assay. SW620 cells were plated out onto 96-well plates in complete medium. The cells were pretreated with or without Sel-
CID-3 at 37°C/5% CO₂ for 1 h. The cells were then stimulated with or without forskolin (10 μM, 0.2% final DMSO) for 1 h. The cells were lysed with HCl (0.1 N) to inhibit phosphodiesterase activity, and the plates were frozen at −20°C. The cAMP produced was measured using cAMP (low pH) Immunoassay kit (R&D Systems).

Cell Cycle, Apoptosis and Caspase 3 Analysis. DNA content analysis was performed using PI staining. Cells were fixed with 70% ethanol overnight before the addition of PI staining solution (50 μg/ml PI and 100 units/ml RNaseA). On analysis, a singlet gate was drawn on a FL-3 width versus FL-3 area dot plot to exclude any aggregates of two or more cells. Cell cycle was analyzed on FL-3 height histograms, and apoptosis was analyzed on two parameter forward scatter versus FL-3 height dot plots. Ten thousand gated events per sample were acquired on a Becton Dickinson FACScan using CellQuest software and analyzed using EXPO 32 and Multicycle software (Beckman Coulter, High Wycombe, United Kingdom).

For caspase 3 determination, cells were collected and incubated for 1 h with a peptide substrate for caspase 3 that has been conjugated to two fluorophores (PhiPhiLux). After washing, caspase 3 activation was determined by flow cytometry. PhiPhiLux fluorescence is detected in FL-1, and for each sample 10,000 cellular events were acquired.

Western Blot Analysis of bcl-2 Family Proteins. Cells were collected, washed in ice-cold PBS and cell extracts prepared. Pellets were resuspended in cold buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 30 mg/ml leupeptin, 5 mg/ml aprotinin, and 5 mg/ml pepstatin A; all from Sigma] and incubated on ice for 5 min. Ten % NP40 was added, followed by KCl. After centrifugation, 20% glycerol was added to the supernatant. Protein concentrations were determined by Bradford assay (Bio-Rad, Hemel Hempstead, United Kingdom).

Cell extracts (25 μg) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Amersham, Amersham, United Kingdom) which were blocked, washed and incubated with specific antisera. Antibodies used were mouse monoclonal antibody to bcl-2, a rabbit polyclonal antibody to bax, a rabbit polyclonal antibody to bcl-xL (all from Santa Cruz Biotechnology, Santa Cruz, CA), and a mouse monoclonal antibody to bak (Calbiochem). Transfer of the proteins onto a PVDF membrane was followed by repeated probing, stripping of the membrane, and reprobing with each of the antibodies, and was carried out as recommended by the manufacturers. Immunodetection was carried out using the enhanced chemiluminescence detection system with horseradish peroxidase-conjugated sheep antimmunoglobulin or donkey antirabbit immunoglobulin secondary antibodies (all from Amersham).

Animal Tumor Xenograft Model. Cultures of BxPC-3 cells were grown to subconfluency in complete medium and viable cells (5 × 10⁵ in PBS) were injected s.c. into the right flank of female athymic nu/nu mice (Harlan, Melton, United Kingdom). When tumors reached 1 × 1 cm, mice were randomized into two groups and were given (i.p./daily) SelCID-3 (10 mg/kg) or vehicle (PBS) alone. Animals were treated for an additional 22 days. Mice were weighed and tumor size/volume calculated using a vernier caliper every 3 days. Data were analyzed using repeated measures ANOVA. For statistically significant observations the data, were further analyzed using a Student-Newman-Keuls test (Primer statistical software). Results were considered statistically significant at P < 0.05 and were expressed as group means with SEs.

Evidence of apoptosis in vivo was obtained using formalin-fixed, paraffin-embedded sections stained for TUNEL and detected by an antidigoxigenin antibody conjugated with peroxidase. Apoptotic nuclei were identified by the presence of dark brown staining. The apoptotic index was defined as the percentage of apoptotic nuclei on total number of tumor cells evaluated in a minimum of 10 fields (×400).

RESULTS

SelCID-3 Inhibits Growth/Viability and Induces G₂-M Arrest in a Variety of Tumor Cell Types. Initial characterization indicated that SelCID-3 is the most effective compound tested at inhibiting the growth of the tumor lines in vitro (Fig. 1). The activity of each compound was consistent within each tumor type. Thus, viability of colorectal and pancreatic tumor cells were preferentially reduced by SelCID-3. Treatment of PBMCs and normal fibroblasts with SelCID-3 had little or no effect on cell viability (data not shown).

The Differential Effect of SelCID Analogues on Tumor Cell Growth/Viability Appears Independent of PDE4-inhibitory Activity and Is Not Caused by cAMP Elevation. The ability of SelCID analogues to inhibit PDE4 provides a possible mechanism for the growth-inhibitory activity of these compounds. We used three compounds that were each a log different in their IC₅₀ values for PDE4 inhibition (SelCID-1, ~3.0 μM; SelCID-3, ~0.3 μM; SelCID-2, ~0.03 μM). Because SelCID-2 is far less effective as an antitumor agent, it is likely that SelCID-3-mediated activity is not caused by PDE4 inhibition.

We then investigated whether the effect of SelCID-3 on SW620 cell viability was associated with cAMP elevation. The data shown in Fig. 2 shows that SelCID-3 induces intracellular cAMP only in the presence of forskolin (an activator of adenylate cyclase). However, inhibition of cell viability by SelCID-3 was apparent without the addition of forskolin, indicating that the elevation of cAMP was not required.

Induction of G₂-M Arrest by SelCID-3 Precedes Caspase 3-dependent Apoptosis of Tumor Cells. Analysis of DNA content was used to determine whether cell cycle arrest was induced by SelCID-3. Cell cycle data indicates that colorectal (SW620), pancre-
atic (BxPC-3), prostate (PC-3), and, to a lesser extent, melanoma (MJT-3) tumor lines have a significant population of cells arrested in G2-M on treatment with SelCID-3 compared with DMSO alone (Fig. 3). This is similar to the inhibition of tumor cell growth observed using the MTS assay. Furthermore, the non-neoplastic adherent human fibroblast line HS 27 was unaffected by SelCID-3.

We then investigated whether cell cycle arrest was apparent before the apoptosis of SelCID-3-treated cells. Fig. 4 shows SW620 cells treated with SelCID-3 for 24 h undergoing extensive G2-M arrest. After 48 h, there was a large increase in apoptotic cells identified as subcellular populations with decreased DNA content (FL-3) and size (forward scatter). From this analysis, it was clear that these dying cells were derived mainly from the arrested G2-M population and also from cells that were in G1. We also detected apoptosis of colorectal and pancreatic tumor cells by SelCID-3 using Annexin V staining and cell death ELISA methods (data not shown). SelCID-3 (up to 100 μg/ml) did not induce apoptosis of normal PBMCs in long-term cultures (up to 5 days; data not shown).

We next set out to determine whether the induction of SW620 cell apoptosis by SelCID-3 is mediated by the activation of caspase 3, an important effector caspase. The induction of caspase 3 was seen at 24 h and was greatly increased at 48 h (Fig. 5A). This closely correlates with the detection of apoptotic cells. Immunoblot analysis confirmed the detection of both the activated form of caspase 3 and its subsequent poly(ADP-ribose)polymerase cleavage product (data not shown).

Inhibition of caspase 3 activation totally prevents SelCID-3-induced apoptosis (Fig. 5B). This was indicated by the lack of cells in sub-G1 and sub-G2-M in the presence of the caspase inhibitor. However, cell cycle arrest was clearly not affected by caspase inhibition as indicated by the large G2-M population.

**Tumor Cell Apoptosis Is Associated with Increased Expression of Pro-apoptotic bak/bax and Reduced Expression of Anti-apoptotic bcl-2.** We found that SelCID-3 induced major changes in tumor cell expression of bcl-2 family proteins (Fig. 6). Expression of bcl-2 was strongly reduced by SelCID-3 (to 37% of control) in SW620 (colorectal) cells, although it was either undetectable or unaffected in other lines. bcl-xL was undetectable in all lines. Pro-apoptotic proteins bak (11.4-fold) and bak (3.8-fold) were increased by SelCID-3 treatment of SW620 cells. T3M-4 (pancreatic) cells showed increased expression of bak (3.2-fold) and bak (1.9-fold). DU-145 (prostate) cells showed increased expression of bak (6.8-fold), and MJT-3 increased expression of bak (2-fold).

For all of the colorectal and pancreatic lines, increased bax:bcl-2 and bak:bcl-2 ratios were observed: for SW620, bax:bcl-2 (untreated, 2.8:1) inactivated by SelCID-3 (1.4:1). The large increase in the bax:bcl-2 and bak:bcl-2 ratios observed with SelCID-3 treatment may be an explanation for the induction of apoptosis in these tumor cell lines.
0.1; +SelCID-3, 3.8) and bak:bcl-2 (untreated, 0.3; +SelCID-3, 3.0); for LoVo, bak:bcl-2 (untreated, 1.4; +SelCID-3, 10.4); for T3M-4, bax:bcl-2 (untreated, 1.5; +SelCID-3, 5.1) and bak:bcl-2 (untreated, 2.3; +SelCID-3, 7.6); and for BxPc-3, bax:bcl-2 (untreated, 1.7; +SelCID-3, 15.2); bak:bcl-2 (untreated, 2.0; +SelCID-3, 3.6).

Therefore, in the most SelCID-3-sensitive lines, there was increased expression of bak and bax alongside decreased bcl-2 expression. In slightly less SelCID-3-sensitive lines, there was an increase in one or the other (but not both) proapoptotic proteins and no effect on bcl-2. Strongly increased bak and bax expression could be detected as early as 8 h after SelCID-3 treatment of SW620 cells (data not shown).

SelCID-3 is Effective as an Antitumor Agent in the Treatment of a Xenotransplantation Model of Cancer. SelCID-3 could inhibit the growth of BxPc-3 tumor cells in vivo. Mice with established tumors were separated into two groups and treated daily with either SelCID-3 (10 mg/kg daily, i.p.) or PBS until the termination of the experiment at day 32. Control mice rapidly developed large tumors. In contrast, mice that were treated with SelCID-3 had greatly reduced tumor mass (Fig. 7). The difference between the groups was apparent at day 20 and reached significance ($P < 0.05$) at day 26 and beyond.

We also detected TUNEL-positive apoptotic nuclei in formalin-fixed, paraffin-embedded sections from SelCID-3-treated mice taken 32 days after tumor challenge (Fig. 7). Apoptosis was significantly increased ($P < 0.01$) in SelCID-3-treated mice (apoptotic index, 66.9 ± 3.5) compared with untreated control mice (apoptotic index, 28.1 ± 1.9). In addition, no SelCID-3-associated effects on morbidity or mortality were noted, which indicated that this compound was well tolerated.

DISCUSSION

The results from this study identify SelCID-3 as representing a novel subclass of the SelCID series of thalidomide analogues with potent activity against a variety of solid tumor cell lines. In contrast to its effect on tumor cells, we found that SelCID-3 is non-cytotoxic when cultured with normal PBMCs or non-neoplastic human adherent fibroblasts. Tumor cell viability was totally inhibited in some tumor types, and this was shown to be caused by the early induction of G2-M cell cycle arrest and subsequent apoptosis within 24–48 h of treatment with SelCID-3. The effects of this analogue on cell viability and cell cycle arrest are most pronounced in the colorectal and pancreatic tumor lines. Interestingly, the identical sensitivities of the colorectal lines to SelCID-3 suggest that, in this context, p53 is not involved in the apoptotic process (LoVo, wild-type p53; SW620 mutant p53). This may be
important clinically because many advanced cancers have p53 mutations. Therefore, agents that are able to induce apoptosis regardless of p53 status may be good chemotherapeutic options.

The effect of SelCID-3 on SW620 cells is in contrast to the documented effect of IMiD analogues in the treatment of myeloma cells in which G1 arrest, but not apoptosis, is prevalent (9). In this regard, we have shown that SelCID-3 is able to induce apoptosis in myeloma cells and may be relevant in the clinical context when discontinuation of treatment might otherwise enable the release of arrested cells and renewed proliferation.

Importantly, the antitumor effect of SelCID-3 is unrelated to the cAMP-elevating properties of the SelCID compounds. SelCID-3 was able to elevate cAMP only in the presence of forskolin (an activator of adenylate cyclase), whereas growth inhibition was apparent without the addition of forskolin. Also, SelCID-2, with far greater anti-PDE4 activity, was ineffective as an antitumor agent. Furthermore, our unpublished observations indicate that SelCID-3 induces myeloma cell apoptosis, whereas a recent study has shown that a number of

\[ \text{A} \]

\[ \text{B} \]

Fig. 5. A, caspase 3 activation is induced by SelCID-3 treatment (50 μg/ml) of SW620 colorectal tumor cells. Flow cytometric analysis was performed on cells stained with a cell-permeable substrate of caspase 3 (PhiPhiLux). The kinetics of caspase 3 induction correlates with the induction of apoptosis in this cell line. B, caspase 3 inhibition prevents SelCID-3-induced apoptosis but not cell cycle arrest of SW620 cells. Z-DEVD-FMK was added twice during the experiment: at 24 h before and also concurrent with SelCID-3 treatment (50 μg/ml). Apoptotic sub-G1 and sub-G2-M cells are derived from G1 and G2-M populations, respectively. However, in the presence of the caspase-3-specific inhibitor, the apoptotic populations were not induced, although the G2-M-arrested cells are still evident.

Fig. 6. SelCID-3 alters the expression of pro- and antiapoptotic proteins. The expression of proapoptotic (bak, bax) and antiapoptotic (bcl-2) proteins was assessed by immunoblot. A, colorectal (SW620), B, pancreatic (T3M-4), C, prostate (DU-145), and D, melanoma (MJT-3) cells were treated with or without SelCID-3 (50 μg/ml) for 24 h and cell lysate prepared for analysis. The immunoblots shown have been equilibrated for protein loading, and each was probed, stripped, and reprobed with each of the antibodies to further ensure continuity of protein loading. The data shown are representative of two separate experiments.

\[ 4 \] J. B. Marriott, manuscript in preparation.

\[ 5 \] Unpublished observations.
other SelCID analogues (which did not include SelCID-3) had minimal effect on myeloma cells (9). Therefore, SelCID-3 represents a novel subgroup within the SelCID group of thalidomide analogues. SelCID-3 has profound effects on the expression of the bcl-2 family proteins, and these were most apparent in tumor lines in which SelCID-3 had the greatest effect. In these cell lines, the ratios of bak:bcl-2 and bax:bcl-2 were strongly increased. However, in less affected cells there was an increase in only one or the other (but not both) pro-apoptotic proteins and no effect on bcl-2. The role of bak/bax in the induction of tumor apoptosis is well established (25). For example, patients with colorectal carcinoma have reduced expression of bak (26), and its overexpression has been shown to mediate caspase-dependent apoptosis (27). Conversely, the overexpression of anti-apoptotic proteins, such as bcl-2, is associated with chemoresistance or poor prognosis in a variety of solid-tumor cell types (26, 28). Therefore, inhibition of bcl-2 may be particularly relevant in the treatment of patients whose conditions are refractory to chemotherapeutic drugs.

Considering the importance of these cellular homologues in the regulation of apoptosis, we think it is possible that the pro-apoptotic effect of SelCID-3 on the various tumor cell types is dependent on its ability to regulate the expression of these proteins. However, this remains unproven, and we have not attempted to investigate the detailed mechanism(s) of action in this study. Therefore, although we have also found that the inhibition of the key effector caspase (caspase 3) prevented SelCID-3-induced apoptosis, we have not investigated the relationship between caspase activation and the alterations in bcl-2 family protein expression. However, our data suggest that, in SW620 cells, the effect of SelCID-3 on bcl-2 family proteins precedes caspase 3 activation.

Our data, showing strong inhibition of tumor growth (and the induction of apoptosis) by SelCID-3 in a preclinical pancancreatic cancer xenograft model, further supports the investigation of SelCID-3 as an anticancer agent. We have also tested several structural analogues of SelCID-3, and these appear to have similar or improved activity against solid tumor and myeloma cell lines in vitro.6

In conclusion, we have identified a novel class of thalidomide analogue that induces G2-M cell cycle arrest and caspase 3-dependent apoptotic death in a variety of solid-tumor cell lines. This is independent of PDE4-inhibitory activity associated with SelCID analogues and correlates with the early induction of the proapoptotic proteins bak and bax, and, in some cases, with the inhibition of antiapoptotic bcl-2. Furthermore, the effect on these proteins is tumor type-specific and is more apparent in the most SelCID-3-sensitive lines. Our results suggest that SelCID-3 represents a novel antitumor agent that is distinct from thalidomide and previously characterized IMiD analogues and that has therapeutic potential against a range of solid tumors, including those with mutant p53. Therefore, this activity potentially extends to the treatment of patients with advanced tumors resistant to standard chemotherapy.

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