Abstract. Survival of patients with pancreatic cancer remains poor due to inadequate chemotherapeutic options. Sansalvamide A, a cyclic depsipeptide produced by a marine fungus, has demonstrated significant anticancer activity. We previously observed antiproliferative effects in a series of sansalvamide A analogs in pancreatic cancer cells, one of which was further evaluated in this study. Two human pancreatic cancer cell lines (AsPC-1 and CD18) were incubated with increasing concentrations (10-50 μM) of the sansalvamide analog. Cell proliferation was then measured by thymidine incorporation and cell counting, and cell cycle analysis was determined by flow cytometry. Western blot analysis was used to evaluate expression of cyclin D1, cdk4, cdk6, cyclin E, cyclin A, cdk2, and p21. Sansalvamide caused G1 phase cell cycle arrest in both cell lines, and Western blot analyses demonstrated up-regulation of p21, down-regulation of cyclins D1, E, and A, and cdk4, consistent with G0/G1 cell cycle arrest. Cumulatively the results show that Sansalvamide A attenuates pancreatic cancer cell growth and represents a potential anticancer therapy.

Pancreatic cancer patients continue to have a poor prognosis due to late diagnosis and limited treatment efficacy (1). Currently, only 20% of pancreatic cancer patients undergoing chemotherapy respond to treatment (2, 3). Clearly, there is an urgent need for agents with improved efficacy against pancreatic cancer.

One promising source of anticancer agents is marine microorganisms (4, 5). Sansalvamide A (Figure 1) is produced by a fungus of the genus Fusarium living on the marine plant Halodule wrightii found on Little San Salvador Island, the Bahamas (6). Sansalvamide A is a cyclic five member depsipeptide that acts to inhibit topoisomerase I (7). Using the National Cancer Institute’s 60 cancer cell line panel, sansalvamide A was found to have significant antiproliferative effects (8). As is common for naturally occurring compounds, analogs of sansalvamide have been created to increase their potency and reduce their cytotoxicity. Previous studies have shown that there are significant effects of these analogs on colon, prostate, and breast cancer, and melanoma (9-16). Liu et al. previously produced twelve analogs through N- methylation and para-bromination, and the efficacy of these analogs was evaluated based on their effects on DNA synthesis (17). Additional experiments revealed that some sansalvamide analogs had higher potency compared to others in addition to characteristically low cytotoxicity. In a prior study, we demonstrated that a highly potent sansalvamide analog, sansalvamide 11, inhibited pancreatic cancer cell growth (18).

In the present study, we evaluated whether another highly potent analog, sansalvamide 12 (Figure 1), would have similar antiproliferative effects in two pancreatic cancer cell lines. Furthermore, we performed Western blot analysis for specific cell cycle phase proteins after sansalvamide 12 treatment.

Materials and Methods

Cell lines and cell culture. The two human pancreatic carcinoma cell lines, AsPC-1 and CD18, were purchased from the American Type Culture Collection (Manassas, VA, USA). AsPC-1 and CD18 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma Chemicals, St. Louis, MI, USA), supplemented with 10%
Cells were grown to 50% confluence in T25-cm² flasks (BD Assay of cellular DNA content by flow cytometry. Assay (Guava Technologies Inc, Hayward, CA, USA). Cells were trypsinized and counted using Guava Technologies' ViaCount Control cells were treated with DMSO. At each time point, the cells were then replaced with fresh serum-free medium containing sansalvamide 12 at 20 μM, which was then followed by treatment with sansalvamide 12 at 20 μM, for 24 hours. There was a significant increase in the percentage of total cells in the G0/G1 phase in cells treated with sansalvamide 12 (Figure 2a). For subsequent experiments, the lowest effective concentration of sansalvamide, 20 μM, was used. CD18 and AsPC-1 cells were treated with sansalvamide (20 μM) for 24, 48 and 72 hours and counted using Guava Technologies' ViaCount Assay. In both cell lines, sansalvamide treatment induced significant growth arrest at all three time points [(72 h, p<0.001) Figure 2b].

Sansalvamide causes G0/G1 cell cycle arrest. Cell cycle analysis was investigated by flow cytometry following sansalvamide treatment of both CD18 and AsPC-1 cell lines for 24 hours. There was a significant increase in the percentage of total cells in the G0/G1 phase in cells treated with the analog 12, CD18 [84% vs. 75% (p<0.05) Figure 3a] and AsPC-1 [80% vs. 70% (p<0.05) Figure 3b], compared to serum-starved control cells.

Sansalvamide reduces cyclin D1, cdk4 and 6, and increases p21 protein expressions. We next performed Western blotting and examined the protein expression levels of the G0/G1 phase proteins, cyclin D1, cdk4 and cdk6. In both CD18 and AsPC-1 cell lines, protein expression of cyclin D1 significantly decreased (p<0.001) after 24 hours of treatment with sansalvamide 12 (Figure 4a). Furthermore,
sansalvamide 12 induced down-regulation of cdk4 (Figure 4b). The change in cdk4 expression was statistically significant after 24 hours of treatment in both CD18 \((p<0.001)\) and AsPC-1 \((p<0.001)\) cell lines. The expression of cdk6 was significantly reduced in the CD18 cell line \((p<0.01)\) and there was a downward trend in AsPC-1 cells after 24 hours (Figure 4c). In addition, Western blot analysis showed that treatment of cells with sansalvamide 12 resulted in the up-regulation of cyclin-dependent kinase inhibitor p21. Induction of p21 (Figure 4d) was shown to be significant in both CD18 \((p<0.05)\) and AsPC-1 \((p<0.05)\) cell lines at the 24 hour time point.

Sansalvamide down-regulates cyclin A and cyclin E but not cdk2 protein expression. Flow cytometric analysis also revealed that there was a significant decrease in the number of cells accumulated in the S-phase (Figure 3a and 3b) concomitant with the G1 arrest. Therefore, we also investigated the levels of cyclins E, A and cdk2 by Western blotting. After 24 hours of treatment with sansalvamide 12, both cyclin E (Figure 5a) and cyclin A (Figure 5b) protein expression decreased in both CD18 and AsPC-1 cells \((p<0.05)\). However, cdk2 protein expression showed no significant change following treatment with the analog at any time point (Figure 5c).

Discussion

Pancreatic cancer is the fourth most common cause of cancer death in the United States, with less than a five percent five-year survival rate (20). Current chemotherapeutic agents used for the treatment of pancreatic cancer have a minimal effect on the prolongation of survival. Thus, more effective agents are needed (21). Sansalvamide A is a promising bioactive molecule that has many advantages due to its cyclic structure, such as a more stable three-dimensional structure and a longer half-life than similar linear peptides (22). While the anticancer mechanisms of this drug remain unclear, previous studies have shown that sansalvamide A acts to inhibit topoisomerase in the poxvirus molluscum contagiosum, which may in part account for its antitumor effects (7). Based on the natural structure of sansalvamide A, many analogous molecules have been synthesized for further investigation (9,}

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**Figure 2.** Sansalvamide causes a concentration and time-dependent decrease in cell proliferation. A, Cell proliferation as shown by thymidine incorporation decreases after cells were serum starved for 24 h and then treated for 24 h with different concentrations of sansalvamide 12. B, Cell count in CD18 and AsPC-1 cell lines decreases following treatment with sansalvamide 12 (T) at different time points. \(*p<0.05, **p<0.01, ***p<0.001, Data are representative of three experiments. \)**\**p<0.001; \**\*p<0.01; \*p<0.05 compared with control (cont, C); Bonferroni’s multiple comparison test.**
Figure 3. Sansalvamide causes a G_0/G_1 cell cycle arrest. A, G_1 cell cycle arrest in CD18 and AsPC-1 cell lines following treatment of cells with 20 μM sansalvamide 12 for 24 h compared to untreated cells. *p<0.05 compared with control by paired t-test. Data are representative of three experiments. B, The corresponding flow cytometric graphs for CD18 and AsPC-1. Similar results were seen in all three experiments.

Figure 4. Sansalvamide down-regulates cyclin D1, cdk4, and cdk6 protein expression and increases p21 expression. CD18 and AsPC-1 cells were treated with 20 μM of sansalvamide for 6, 12, and 24 hours. Twenty-four hour treatment caused the down-regulation of cyclin D1 (a) in both CD18 and AsPC-1 cells at all three time points. cdk4 (b) significantly decreased in CD18 cells at 12 and 24 hours and AsPC-1 at 24 hours. cdk6 (c) was shown to be significantly decreased at 24 hours in CD18 but not in AsPC-1 cells. The expression of p21 (d) was up-regulated in both cell lines after treatment with sansalvamide for 24 hours. Representative Western blots are shown under their corresponding optical densities as determined by image J (NIH). Refer to Figure 5c for associated GAPDH expression. This figure is representative of a minimum of three experiments. **p<0.01 ***p<0.001, as compared with control, Bonferroni’s multiple comparison test.
Other studies have shown that the effect of different analogs of sansalvamide A on a variety of cancer types, including drug-resistant colon cancer, matches the effect of current chemotherapeutic drugs being used. The results of these studies demonstrate that sansalvamide A is a promising structure for a new class of antitumor drugs (9-17, 24).

In the current study, we used a different analog, sansalvamide 12, and determined the appropriate concentration to use on the pancreatic cancer cells by using methyl-[3H] thymidine incorporation. The data from cell count experiments demonstrated that the number of cells in cultures treated with sansalvamide 12 did not decrease further from 24 hours to 72 hours, while untreated cells increased in number over the same period of time (Figure 2b). This result led us to speculate that the current sansalvamide analog primarily works by preventing the pancreatic cancer cells from proliferating rather than by inducing apoptosis. This view was supported by the results of flow cytometry performed on two pancreatic cancer cell lines, CD18 and AsPC-1, which showed an accumulation of cells in the G0/G1 phase of the cell cycle.

After demonstrating that sansalvamide 12 induces G0/G1 cell cycle arrest, we further investigated the mechanism of growth arrest. We first investigated the proteins involved in G1 phase and observed a decrease in cdk4, cdk6, and cyclin D1 in addition to an increase in p21 protein expression (Figure 4). These changes are consistent with our flow cytometric data showing G0/G1 arrest (25, 26). Concomitant with an increase in the number of cells in G1, there was a decline in cell number in the S-phase. Therefore, we examined the levels of cyclins E, A and cdk2. Both cyclin E and A were significantly reduced on treatment with sansalvamide 12 (Figure 5). However, cdk2 levels were not significantly changed, although there was a trend towards a decrease in expression at 24 hours of treatment with sansalvamide 12. Figure 6 summarizes the mode of action of sansalvamide 12 in inducing G0/G1 cell cycle arrest in these two pancreatic cancer cell lines.

The gold standard of pancreatic cancer treatment, gemcitabine, has minimal efficacy in comparison to chemotherapeutic treatments in other types of cancer. The limited success of single-agent chemotherapy for pancreatic cancer has led to the study of combination treatments with gemcitabine (27, 28). The study of analogs of sansalvamide A in combination with gemcitabine could be carried out to obtain a better efficacy for patients. We are currently in the process of conducting in vivo studies using sansalvamide analogs to expand the in vitro experimental data and determine possible associated toxicity. The development of better chemotherapeutic agents is critical for the improvement of the prognosis for patients with pancreatic cancer. More additional experiments are needed.
needed to fully explain the anticancer effects observed. From the current study, we conclude that sansalvamide A inhibits proliferation of pancreatic cancer cells and deserves attention for development in clinical therapeutic approaches.

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References


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