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Combined cytotoxic action of paclitaxel and ceramide against the human Tu138 head and neck squamous carcinoma cell line

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Abstract *Purpose*: Paclitaxel, a chemotherapeutic agent used in the treatment of recalcitrant ovarian and breast as well as other neoplasms, is being investigated for the treatment of squamous cell carcinoma of the head and neck. Our previous studies have demonstrated that exogenously added ceramide enhances apoptosis in paclitaxel-exposed human leukemic cells. In this study, we showed that exogenous ceramide augmented paclitaxel-induced apoptosis in Tu138 cells in vitro when added simultaneously in combination with the paclitaxel. *Methods*: The combined cytotoxic effects of paclitaxel and ceramide exposure against Tu138 cells were assessed by an MTT dye assay, cell cycle analysis, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay, and isobologram analysis for synergistic activity. *Results*: The MTT dye assay results indicated augmentation of time- and concentration-dependent paclitaxel-mediated cell cytotoxicity by simultaneous ceramide treatment. Paclitaxel treatment of Tu138 cells also resulted in an accumulation of cells in the G_2-M phase of the cell cycle. This paclitaxel-mediated G_2-M phase accumulation decreased significantly with the addition of ceramide, indicating that combined paclitaxel/ceramide treatment resulted in the elimination of Tu138 cells from the S and/or G_2-M phases of the cell cycle. Furthermore, ceramide enhancement of paclitaxel-mediated apoptosis was also detected by the TUNEL assay. *Conclusion*: Our results suggest that paclitaxel/ceramide combination therapy may be an attractive alternative to conventional methods of chemotherapy for head and neck cancer, and should be further explored.

Key words Paclitaxel · Ceramide · Squamous cell carcinoma

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Introduction

Squamous cell carcinoma of the head and neck is a particularly aggressive type of cancer, refractory to many current treatment regimens. Although chemotherapeutic agents have been routinely utilized in combination to arrest tumor cell growth, many have proven unsuccessful. Recent studies have demonstrated the promising efficacy of combination treatment with paclitaxel [17]. Paclitaxel is a chemotherapeutic agent that prevents microtubule depolymerization resulting in the arrest of proliferating cells in the G_2-M phase of the cell cycle and leading to cell death [39]. Additionally, paclitaxel modulates a number of intracellular events which result in cellular apoptosis and ensuing nuclear degradation, including phosphorylation of Bcl-2 [21], or transient stimulation of c-Jun kinase (JNK) [2], activation of caspases, and cleavage of downstream caspase substrates such as poly-ADP ribose polymerase (PARP) [43].

Paclitaxel has been used alone and in conjunction with radiation and/or chemotherapeutic agents to treat a number of different cancers in clinical trials [18, 24]. Combination therapy with paclitaxel and radiation, cisplatin, or doxorubicin has been shown to induce high clinical response rates in patients with squamous cell cancer of the head and neck [12]. Despite the fact that

paclitaxel has been proven to be effective in the treatment of a number of aggressive types of neoplasms, including head and neck cancer, a major disadvantage of paclitaxel therapy is the broad range of severe hypersensitivity reactions associated with its administration, as well as neurotoxicity and myelosuppression [37]. As a result, numerous recent studies have focused on paclitaxel combination therapy with the ultimate goal of increasing therapeutic efficacy at a lower concentration of paclitaxel thus decreasing its toxicity.

Previous in vitro studies have indicated that ceramide is capable of enhancing paclitaxel-mediated growth inhibition and apoptosis, thus lowering the ED_{50} of paclitaxel and suggesting that its toxic effects could be reduced via dose reduction in vivo [33]. Ceramide, a compound primarily derived endogenously from sphingomyelin, a sphingolipid precursor which comprises part of the cell membrane, has been shown to have numerous regulatory effects on cell function, including cell growth and differentiation, cell cycle arrest, inflammation, and apoptosis [4, 25, 26, 34]. Sphingomyelin hydrolysis occurs after the subsequent activation of acid or neutral sphingomyelinase by any one of several exogenous mediators, including TNFα, endotoxins, interferon-α, IL-1, Fas ligand, CD28, chemotherapeutic agents, heat and ionizing radiation, resulting in elevation of intracellular ceramide levels [22, 28]. Ceramide synthesis de novo has been implicated in lethal responses to several chemotherapeutic agents such as anthracyclines and ara-C [5, 45]. An in vitro model of paclitaxel/ceramide combination therapy is presented here. We demonstrated that apoptosis induced by clinically relevant concentrations of paclitaxel in the human Tu138 head and neck squamous cell carcinoma cell line can be enhanced by ceramide.

Materials and methods

Tumor cell line

Tu138, an adherent head and neck squamous carcinoma cell line, was generously donated by Dr. Gary Clayman's laboratory, University of Texas M D Anderson Cancer Center, Houston, Tx. [10, 31]. Tu138 cells were routinely maintained in T-75 culture flasks (Falcon, N.J.) at a plating cell density of $0.1 \times 10^6/75$ cm^2 surface area in complete DMEM/F-12 culture medium (10 ml) containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Ga.), 2 m*M* glutamine (Gibco, N.Y.), 50 U/ml penicillin, 50 mg streptomycin (Gibco, N.Y.) and 20 m*M* HEPES (Sigma, Mo.) at 37 °C in an atmosphere containing 5% CO_2. Tu138 cell cultures were replenished with fresh complete culture medium and reseeded twice weekly.

Treatment of Tu138 tumor cells with paclitaxel and/or ceramide

Prior to paclitaxel and/or ceramide exposure, Tu138 cells were trypsinized in 0.25% trypsin-EDTA, washed twice in complete DMEM/F-12, and plated in 96-well culture plates at 50×10^3 cells/ml in a final volume of 0.2 ml in complete DMEM/F-12. Cells were incubated in the absence or presence of different concentrations of paclitaxel (0–6000 ng/ml; Bristol Myers Squibb, N.J.) and/or C6 ceramide (*N*-hexanoyl-D-sphingosine, 0–25 μg/ml; Sigma Chemicals, Mo.). The highest paclitaxel concentration utilized was selected to be within the achievable range for clinical treatment. Cells were subjected to (a) tetrazolium-based dye assay of cell survival, (b) flow cytometry analysis of cell cycle progression, and (c) TUNEL assay for the measurement of apoptosis.

MTT assay

Cellular cytotoxicity was measured by the addition of 50 μl of a 0.2% solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye (Sigma Chemicals, Mo.) to Tu138 cells [7] after treatment with paclitaxel and/or ceramide. MTT-treated cultures were then incubated for 4 h at 37 °C. Culture plates were then centrifuged at 300 *g* for 2 min and the culture supernatants removed. MTT formazan crystals formed by cells undergoing coupled respiration were solubilized by the addition of 150 μl DMSO after removal of the culture medium. Optical density was determined spectrophotometrically (Model EL 311, Biotek) at 540 nm.

Cell cycle measurements and TUNEL assay

Tu138 cells were exposed to paclitaxel (600 ng/ml) and/or ceramide (25 μg/ml), plated in six-well culture dishes at 0.5×10^6 cells/ml in complete DMEM/F-12, and incubated at 37 °C for either 24 or 48 h. Cells were then trypsinized and washed in complete DMEM/F-12.

Cell cycle measurements were made following the addition of 0.5 ml 0.1% propidium iodide solution (New Concepts Scientific, Niagara Falls, NY, USA) containing 0.1% sodium citrate and 0.1% NP-40. RNase (1 μg) was added to each sample and cells were incubated at 4 °C for 30 min. Acquisition of at least 1×10^4 cells was recorded using a FACScan (Becton Dickinson, Calif.) flow cytometer. A precalibration DNA QC kit (Becton Dickinson) was utilized. The acquired cells were then subjected to cell cycle analysis using Modfit LT software (Becton Dickinson).

Apoptosis was measured by flow cytometry using an in situ cell death detection kit (Boehringer Mannheim, Ind.).

Results

Paclitaxel- and ceramide-induced cytotoxicity as a function of time of exposure

The aim of the first set of experiments was to observe the cytotoxic effects of paclitaxel and ceramide at different concentrations separately on Tu138 cells as measured by the MTT dye assay. As shown in Fig. 1A, paclitaxel-induced cytotoxicity was 37% and 64% at 600 and 6000 ng/ml, respectively, whereas a 72-h exposure to ceramide at 12.5 and 25 μg/ml resulted in 19% and 46% cytotoxicity, respectively (Fig. 1B). These experiments ($n = 6$) indicated that the ED_{50} values for paclitaxel and ceramide were 1920 ± 1200 ng/ml and 22 ± 5 μg/ml, respectively.

The enhancement of cellular cytotoxicity by paclitaxel and ceramide in combination was evaluated by incubating Tu138 cells with paclitaxel (600 ng/ml) and/or ceramide (25 μg/ml). This paclitaxel concentration is within the clinically achievable range using a 24-h infusion in patients. Cell viability was estimated using the MTT dye assay at 24-h time intervals, concluding at 72 h. As shown in Fig. 2, paclitaxel alone at 600 ng/ml reduced cell viability by from 0.9% at 24 h to 21.8% at 72 h. Treatment with ceramide also reduced cell viability

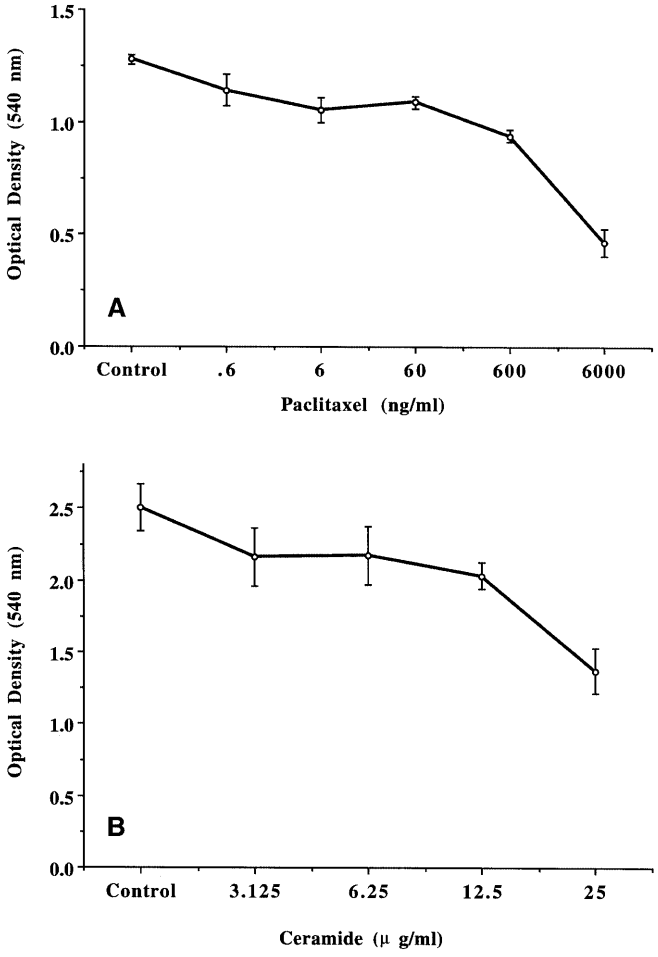


Fig. 1A,B Effect of paclitaxel and ceramide on the growth of Tu138 cells. Tu138 cells (50×10^3/ml) were plated in a 96-well culture plates in the presence and absence of paclitaxel or ceramide for a period of 3 days and then subjected to the MTT dye assay as described in Materials and methods. The *x*-axis represents concentrations of paclitaxel (0–6000 ng/ml; **A**) and ceramide (0–25 μg/ml; **B**) and the *y*-axis represents optical density (540 nm) of live cells that incorporated MTT dye. The averages and standard deviations of triplicate determinations are shown

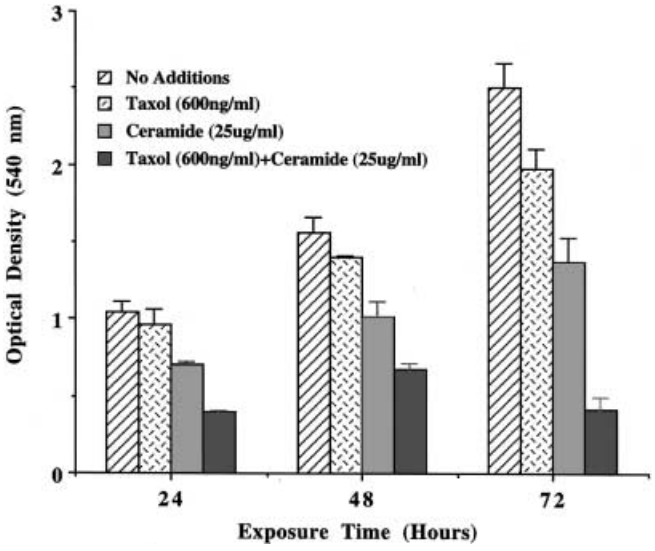


Fig. 2 Time kinetics of the action of paclitaxel and ceramide alone and in combination. Tu138 cells were cultured as indicated in the legend for Fig. 1 in the presence and absence of paclitaxel (600 ng/ml) and/or ceramide (25 μg/ml)

by from 32.7% at 24 h to 54.5% at 72 h. Additionally, combined paclitaxel (600 ng/ml) and ceramide (25 μg/ml) treatment resulted in a 68% reduction in cell viability over ceramide alone during a 3-day incubation period. Enhancement of cytotoxicity was also evident with combination treatment at 24 h (62.0% cell kill), with maximum cytotoxicity observed at 72 h. Thus, the combination of the two agents seemed to result in an enhancement over the additive effect with respect to loss of electron transport function and subsequent loss of cell viability.

The results of 23 different observations of the effects of paclitaxel (600 ng/ml), ceramide (25 μg/ml) and their combination were then subjected to statistical analysis. The mean observed optical density (obtained after different exposures) and the standard deviation were analyzed over the confidence interval with 95% probability. The value of the confidence range was compared by assuming a Student's *t* distribution with the selected exposure conditions. The paired *t*-test demonstrated that the results with the paclitaxel and ceramide combination were significantly different from the results with either paclitaxel or ceramide alone with $>95\%$ probability.

Synergistic interaction analysis by isobologram

To determine whether the cytotoxic effect of the combination of paclitaxel and ceramide against Tu138 cells was synergistic or additive, a quantitative isobologram analysis [8, 15] of the results of six independent experiments in triplicate was performed with various dilutions of paclitaxel (0–6000 ng/ml) and ceramide (0–25 μg/ml) administered separately and simultaneously. A straight line joining points on *x*- and *y*-axes (Fig. 3) represents ED_{50} concentrations of paclitaxel and ceramide as determined from the six independent experiments. Because of the nonlinearity of the dose response curves with either paclitaxel or ceramide alone, a second line plot was generated by calculating the ED_{10} through ED_{50} for both agents alone. The line plot represents the ED_{40} of paclitaxel combined with the ED_{10} of ceramide or paclitaxel ED_{30} plus ceramide ED_{20} and so on. The isobologram using both the straight line approximation and the curve generated from the experimental data to compare the effects of combined additions is shown in Fig. 3. The points to the left of the two line plots that fall within the ED_{50} concentrations indicate supraadditivity of the two agents tested in combination. The isobologram analysis of the six experiments represented as scatter plots indicated supraadditivity. The linear approximation assumption indicated that 21 of 23 experimental points were synergistic. The experimental

determination showed 18 of 23 points to be in the synergistic range.

Paclitaxel- and ceramide-induced modification of cell cycle kinetics

It has been suggested that paclitaxel and ceramide cause cell cycle arrest and apoptosis. To determine whether the combination of paclitaxel and ceramide affects the kinetics of cell cycle progression, the effects of paclitaxel at 600 ng/ml and ceramide at 25 μg/ml (at or near their ED_{50} values) alone and in combination on the cell cycle distribution of cells during growth were evaluated by flow cytometry utilizing the propidium iodide staining technique. Since it was difficult to obtain an adequate number of viable cells from samples exposed to combination treatment for 72-h, cell cycle measurements were made only at 24 and 48 h. Figure 4 demonstrates that paclitaxel was able to block 61.8% (24 h) and 58.2% (48 h) of Tu138 cells in the G_2-M phase of the cell cycle. This contrasts with 7.4% (24 h) and 6.0% (48 h) of untreated control cells. Ceramide added alone did not significantly affect progression through either the G_0-G_1 or G_2-M phases of the cell cycle compared to untreated controls ($n = 4$) at either 24 or 48 h.

In cultures treated simultaneously with paclitaxel and ceramide, G_2-M arrest percentages of 45.6% and 28.4% were observed at 24 and 48 h, respectively, in comparison with paclitaxel-treated cultures. This indicates a loss of 26.3% and 51.3% of the G_2-M population of viable Tu138 cells due to the combined effect of paclitaxel and ceramide in comparison with paclitaxel alone at 24 and 48 h, respectively. A significant loss of the S-phase population was observed only at 48 h. Along with results from the MTT experiment, these findings indicate that cell death occurred either in the G_2-M phase or during the exit from the S-phase of the cell cycle.

Induction of apoptosis by combined paclitaxel/ceramide exposure

Apoptosis is now recognized as an important mechanism for chemotherapy-induced cell kill [27]. Since the MTT dye assay and the measurement of G_2-M cell population cannot determine whether cell loss is the result of apoptosis or necrosis, and since paclitaxel and ceramide when added alone have been shown to induce apoptosis in numerous studies [25, 26], the next series of experiments were directed to determining the mode of cell death induced by combined exposure to paclitaxel and ceramide at 24 and 48 h by the TUNEL assay. As demonstrated in Fig. 5A, treatment with either paclitaxel (600 ng/ml) or ceramide (25 μg/ml) alone resulted in 18.4% and 9.7% apoptosis at 24 h, respectively, whereas treatment with the two agents combined induced 53.7% apoptosis. This reflects a supraadditive response and demonstrates an approximate threefold increase in comparison to the results with paclitaxel alone. At 48 h (Fig. 5B), paclitaxel and ceramide individually induced 54.8% and 13.6% apoptosis, respectively, compared with apoptosis of 7.9% in untreated cells. The percentage of apoptotic cells rose to 84.9% following simultaneous exposure to the two agents in combination, again indicating supraadditivity.

Discussion

We examined the combined effect of paclitaxel and ceramide, two agents with diverse activities, on neo-

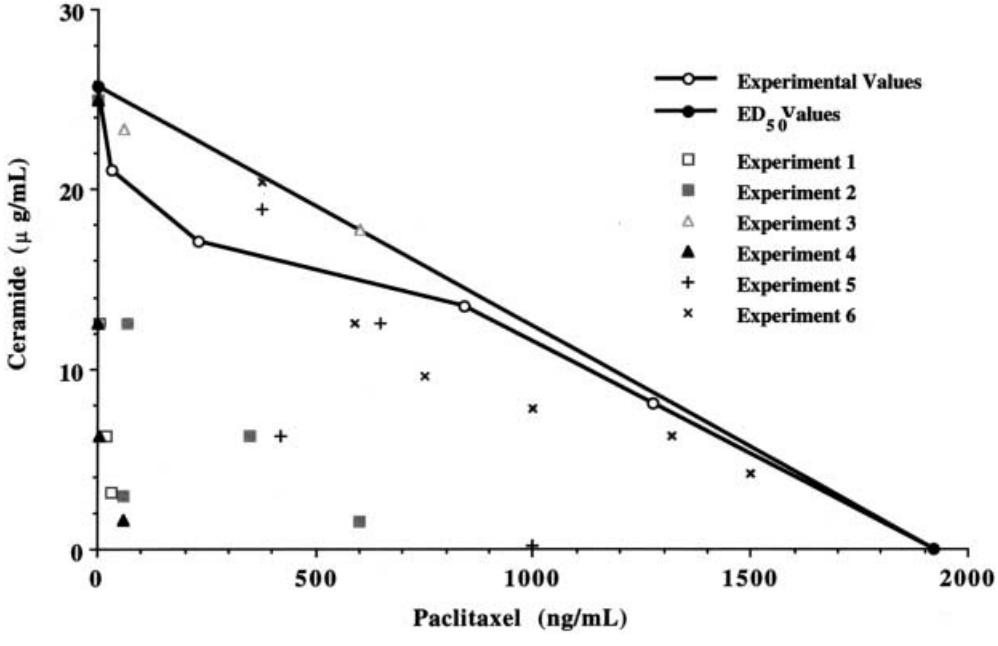
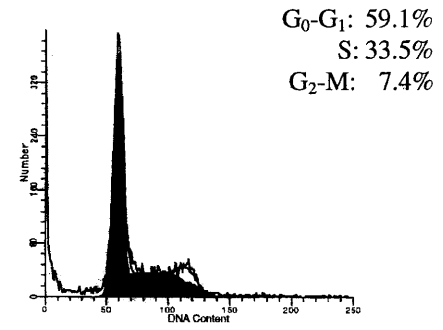
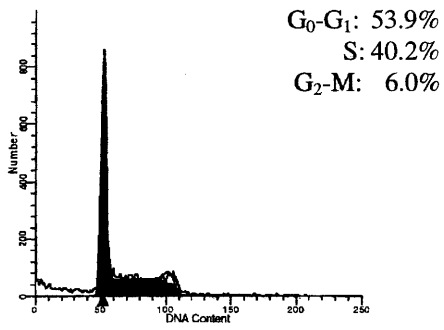


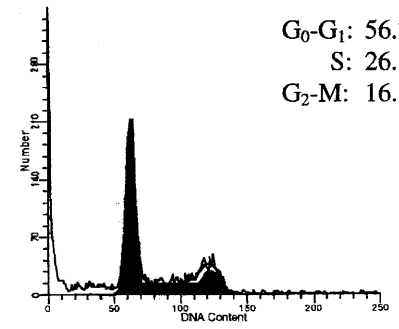
Fig. 3 ED_{50} isobologram analysis of the action of paclitaxel and ceramide in combination. Isobolograms were constructed by determining individual ED_{50} values from experiments ($n = 6$) performed at various concentrations of paclitaxel and ceramide by best fit analysis. The line plot was generated by calculations of ED_{10} through ED_{50} values for both paclitaxel and ceramide. The points on the scatter plot are the calculated ED_{50} values from seven independent experiments involving the exposure of Tu138 cells to paclitaxel and ceramide in combination



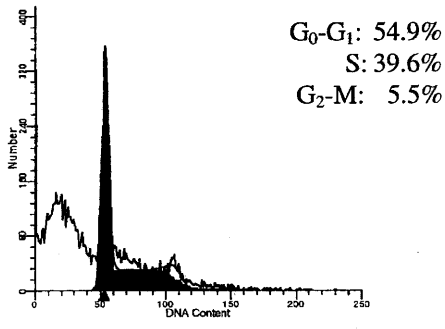
Control - No Additions (24 hours)



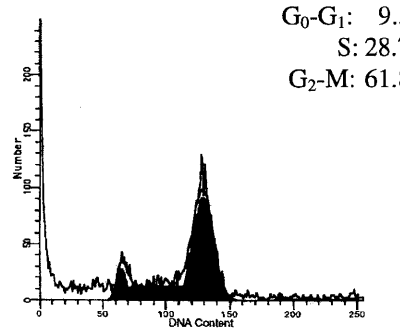
Control - No additions (48 hours)



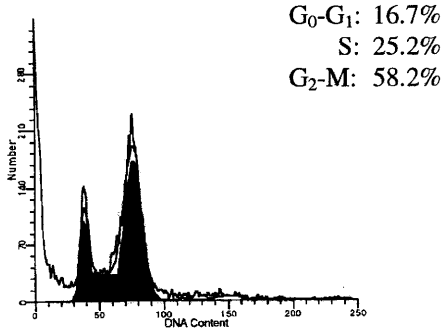
Ceramide 25 µg/ml (24 hours)



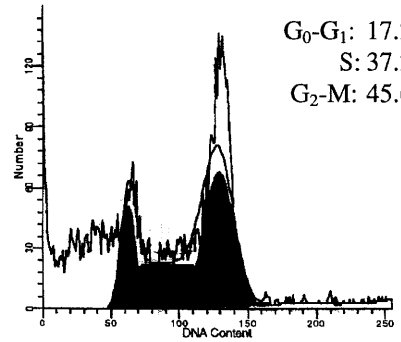
Ceramide 25 µg/ml (48 hours)



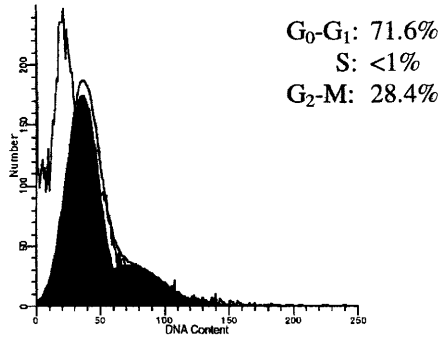
Paclitaxel 600 ng/ml (24 hours)



Paclitaxel 600 ng/ml (48 hours)



Ceramide 25 µg/ml (24 hours)
+ Paclitaxel 600 ng/ml



Ceramide 25 µg/ml (48 hours)
+ Paclitaxel 600 ng/ml

Fig. 4 Flow cytometric analysis of the effects of paclitaxel and ceramide alone and in combination on cell cycle progression of Tu138 cells. Tu138 cells at a density of 0.5×10^6/ml were cultured in the presence and absence of paclitaxel (600 ng/ml) and/or ceramide (25 µg/ml) for 24 and 48 hours in six-well culture plates. At the end of the incubation period, cells were trypsinized, washed and subjected to flow cytometric analysis as described in Materials and methods. The analysis of the acquired samples with the use of Modfit software is shown alongside each treatment as the percentage of the population of viable cells in the various phases of the cell cycle

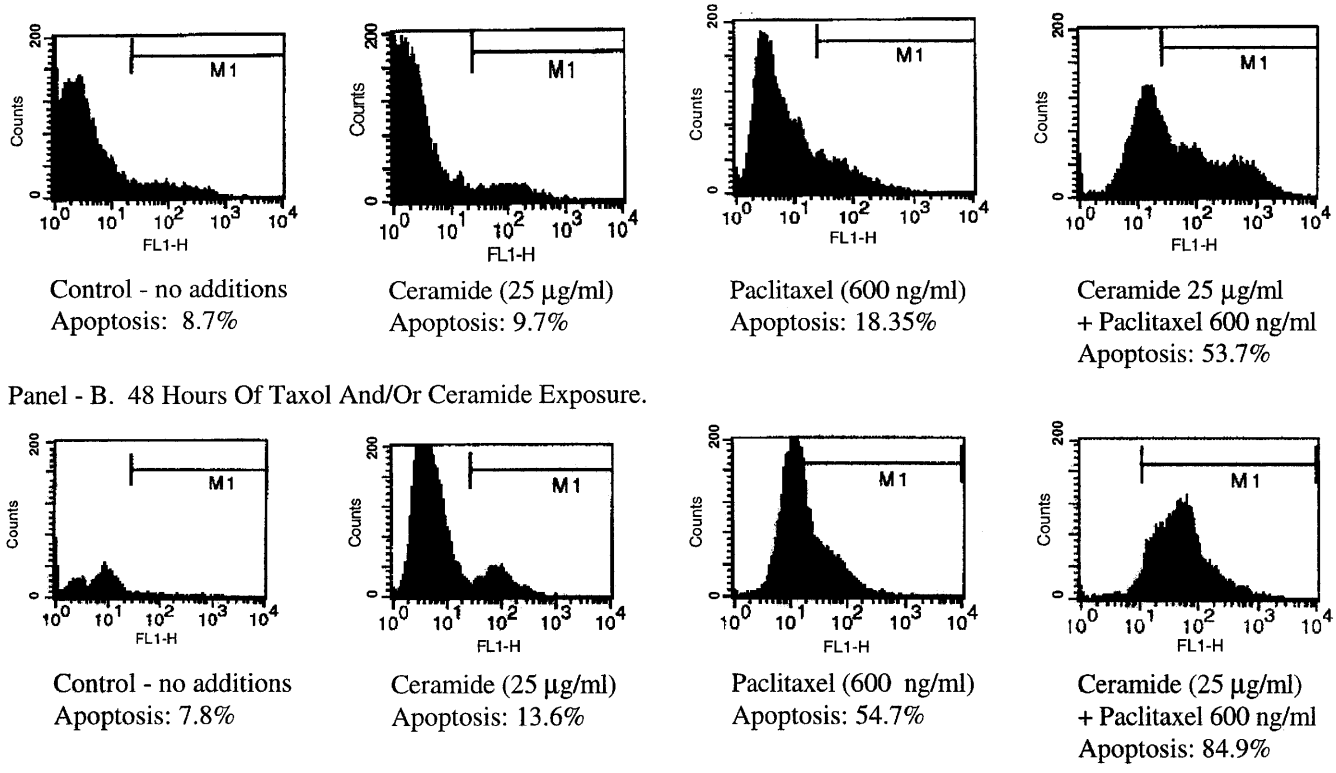


Fig. 5A,B TUNEL assay results showing the kinetics of the induction of apoptosis in Tu138 cells following exposure to paclitaxel and ceramide alone and in combination for 24 h (**A**) and 48 h (**B**). Tu138 cells were cultured with or without paclitaxel (600 ng/ml) and/or ceramide (25 µg/ml) as described in the legend to Fig. 4. The analysis of the acquired samples was based on antibody binding to DNA fragments measured as fluorescence (shown on the *x*-axis, with cell counts on the *y*-axis). For the positive control, Tu138 cells were treated with DNAse for 10 min at room temperature prior to the acquisition on FACScan. The apoptosis percentage shown under each scan was obtained by the use of CELL Quest software (Becton Dickinson, Calif.) and is represented by M1

plastic cell growth inhibition, cell cycle progression, and apoptosis. Paclitaxel has been clearly demonstrated to be an extremely important agent in cancer chemotherapy. It has already been shown to have considerable antitumor activity against high-grade carcinomas and relatively resistant tumors of the head and neck [13, 18, 42, 49]. Our studies confirm that paclitaxel is capable of inducing cell cycle arrest in the head and neck carcinoma cell line Tu138 in the G_2-M phase of the cell cycle, as has been documented in several head and neck cancer cell lines [1, 16, 35]. The finding that paclitaxel is cytotoxic to head and neck carcinoma cells in vitro, as demonstrated by the MTT assay (ED_{50} 1920 ± 1200 ng/ml), also concurs with previous reports of the successful use of paclitaxel treatment in attenuating cell proliferation and promoting cell death [35]. Additionally, the large variation in ED_{50} may be attributed to the proliferation of an asynchronous population of Tu138 cells. Such cell cycle sensitivity and susceptibility to paclitaxel-induced cell death has been demonstrated in ovarian, breast, lung and numerous other cell lines [9, 32, 38, 44].

The exogenous addition of ceramide results in apoptosis in a variety of tumor cell lines [26, 45]. Our current studies of solid tumors of the head and neck indicate that ceramide-mediated cytotoxicity towards Tu138 cells occurs with an ED_{50} of 22 ± 5 µg/ml. In addition, ceramide induced apoptosis in Tu138 cells as measured by the TUNEL assay. This finding contradicts the results of previous studies in which no significant effect of ceramide treatment on cellular apoptosis of leukemic cells has been found [33, 46]. However, a significant effect of ceramide on the cell cycle progression of Tu138 cells was not noted, which concurs with the findings of a pervious study of Jurkat leukemic T-cells [33].

Paclitaxel (600 ng/ml) and ceramide, when added separately, mediated apoptosis of Tu138 cells within 24 h of exposure. However, when the two agents were combined, the observed cytotoxicity was greater than expected for the combination, indicating a synergistic interaction between these two agents. This was confirmed by isobologram analysis of the MTT dye assay data, demonstrating synergy in >78% of the tested experimental concentrations of paclitaxel and ceramide combined. Additionally, a significant reduction of the G_2-M cell population within 48 h following the addition of paclitaxel and ceramide combined was evident from cell cycle analysis. Taken together, the MTT dye and TUNEL assay results seem to suggest that the loss of cell

population was from either the S or the G_2-M phase of the cell cycle, a finding which corroborates the results of our recent study of combined paclitaxel/ceramide treatment in Jurkat cells [33].

The intracellular mechanism of paclitaxel/ceramide-induced apoptosis remains unclear. One possible mechanism of enhancement of paclitaxel-induced cytotoxicity by ceramide is through increased production of intracellular ceramide. Ceramide generation occurs after hydrolysis of sphingomyelin by acid and/or neutral sphingomyelinase enzymes downstream from the TNF receptor, and in turn facilitates apoptosis [5, 6, 34]. Activation of apoptosis has also been shown to occur directly through the activation of ceramide synthase with certain chemotherapeutic drugs such as daunorubicin [5]. Our preliminary data demonstrate that the effects of the combined treatment could not be blocked by the addition of the ceramide synthase inhibitor, fumonosin (data not shown), negating a role for acid or neutral sphingomyelinase.

Additionally, a number of apoptotic mediators through which paclitaxel and ceramide signal individually have been identified. These include receptors such as TNFα and Fas, and mediators including Bcl-2 family proteins, JNK, caspases, and cysteine protease substrates such as PARP [2, 3, 21, 43, 46, 47, 51]. Many other chemotherapeutic agents – for example, doxorubicin – have been shown to mediate apoptosis through the involvement of Fas receptor/ligand system [20], but this has been questioned in recent reports [14, 48]. Additionally, numerous studies have indicated that paclitaxel-induced apoptosis occurs partially as a result of phosphorylation of JNK and Bcl-2 [2, 21, 50]. Furthermore, paclitaxel treatment results in the cleavage of caspase-3 and downstream substrates of caspase-3 such as PARP [42]. Ceramide, like paclitaxel, induces JNK activation and caspase-3 cleavage [3, 51]. Additionally, our preliminary data suggest that caspase-8 is a central mediator for the synergistic execution of apoptosis by paclitaxel and ceramide in human leukemic cells (manuscript in preparation). Therefore, we suggest that the mechanism by which paclitaxel and ceramide mediate a synergistic effect may be via a commonality in downstream signal transduction pathways.

Paclitaxel has been evaluated in combination with cisplatin, doxorubicin and radiation in several studies of head and neck cancer patients with encouraging results [11, 19, 23, 29, 30, 36, 41]. However, the investigation of new treatment modalities for the management of head and neck carcinoma such as combination therapy with ceramide and paclitaxel, which might enhance the therapeutic potential of paclitaxel or allow a reduction of paclitaxel dose and associated toxicities is necessary. Saffingol (L-threo-dihydrosphingosine) at pharmacologically active concentrations in combination with doxorubicin has been investigated in pilot clinical trials and has shown minimal toxicity in patients with confirmed carcinoma [40]. Similarly, our results raise the possibility that paclitaxel/ceramide combination therapy may be an attractive alternative to conventional methods of chemotherapy for head and neck cancer, and should be further explored.

References

1. Ain KB, Tofiq S, Taylor KD (1996) Antineoplastic activity of taxol against human neoplastic thyroid carcinoma cell lines in vitro and in vivo. J Clin Endocrinol Metab 81: 3650
2. Amato SF, Swart JM, Berg M, Wanebo HJ, Mehta SR, Chiles TC (1998) Transient stimulation of the c-Jun-NH_2-terminal kinase/activator protein 1 pathway and inhibition of extracellular signal-regulated kinase are early effects in paclitaxel-mediated apoptosis in human B lymphoblasts. Cancer Res 58: 241
3. Anjum R, Ali AM, Begum Z, Vanaja J, Khar A (1998) Selective involvement of caspase-3 in ceramide induced apoptosis in AK-5 tumor cells. FEBS Lett 439: 81
4. Beilawska A, Linardic CM, Hannun YA (1992) Modulation of cell growth and differentiation by ceramide. FEBS Lett 307: 211
5. Bose R, Verheij M, Haimovitz-Friedman A, Scotto K, Fuks Z, Kolesnick R (1995) Ceramide synthase mediates daunorubicin-induced apoptosis and alternative mechanism for generating death signals. Cell 82: 405
6. Bursh W, Kliene L, Tenniswood M (1990) The biochemistry of cell death by apoptosis. Biochem Cell Biol 68: 1071
7. Carmichael J, DeGraf WJ, Gazdar AF, Minna JD, Mitchell JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 47: 936
8. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effect of multiple drugs and enzyme inhibitors. In: Weber G (ed) Advances in enzyme regulation. Pergamon Press, New York, pp 27–55
9. Chuang L, Lotzova E, Leath J, Cook K, Munkanah A, Morris M, Wharton J (1994) Alterations of lymphocyte microtubule assembly, cytotoxicity and activation by the anticancer drug Taxol. Cancer Res 54: 1286
10. Clayman G, Liu T, Overholt M, Mobley S, Wang M, Janot F, Goepfert H (1996) Gene therapy for head and neck cancer. Arch Otolaryngol Head Neck Surg 122: 48
11. Conley B, Jacobs M, Suntharalingam M, Zacharski D, Ord RA, Gray W, Aisner J (1997) A pilot trial of paclitaxel, carboplatin and concurrent radiotherapy for unresectable squamous cell carcinoma of the head and neck. Semin Oncol 24 [1 Suppl 2]: S2–78
12. Cortes-Funes H, Aisner J (1997) Paclitaxel in head and neck cancer and other tumor types. Semin Oncol 24: 52
13. Creaven P, Rayhavan C, Pendayala D, et al (1995) Phase 1 study of paclitaxel and carboplatin: implications for trials in head and neck cancer. Semin Oncol 22: 13
14. Eischen CM, Kottke TJ, Martins LM, Basi GS, Tung JS, Earnshaw WC, Liebson PJ, Kaufmann SH (1997) Comparison of apoptosis in wild-type and Fas-resistant cells: chemotherapy-induced apoptosis is not dependent on Fas/Fas ligand interactions. Blood 90: 935
15. Elion GB, Singer S, Hichings GH (1954) Antagonists of nucleic acid derivatives. VIII. Synergism in combinations of biochemically related antimetabolites. J Biol Chem 208: 477
16. Elomaa L, Joensuu H, Kulmala J, Klemi P, Grenman R (1995) Squamous cell carcinoma is highly sensitive to taxol, a possible new radiation sensitizer. Acta Otolaryngol (Stockh) 115: 340
17. Forastiere AA (1994) Paclitaxel (Taxol) for the treatment of head and neck cancer. Semin Oncol 21: 45
18. Forastiere AA, Urba SC (1995) Simple agents paclitaxel and paclitaxel tifosfamide in the treatment of head and neck cancer. Semin Oncol 22: 24
19. Fountzilas G, Athanssiadis A, Samantas E, et al (1997) Paclitaxel and carboplatin in recurrent and metastatic head and neck cancer: a phase II study. Semin Oncol 24: S2–65

20. Friesen C, Herr I, Krammer PH, Debatin KM (1996) Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat Med 2: 574
21. Haldar S, Chintapalli J, Croce CM (1996) Taxol induces Bcl-2 phosphorylation and death of prostate cancer cells. Cancer 56: 1253
22. Hannun YA (1994) The sphingomyelin cycle and the second messenger function of ceramide. J Biol Chem 269: 3125
23. Hitt R, Hornedo J, Colomer R, et al (1997) Study of escalating doses of paclitaxel plus cisplatin in patients with inoperable head and neck cancer. Semin Oncol 24: S2–58
24. Holmes FA, Walters RS, Theinault RL, et al (1991) Phase ll trial of Taxol, an active drug in the treatment of metastatic breast cancer. J Natl Cancer Inst 83: 1797
25. Jarvis WD, Kolesnick RN, Fornari FA, Traylor RS, Gewirtz DA, Grant S (1994) Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. Proc Natl Acad Sci USA 91: 73
26. Jayadev S, Liu B, Bielawska AE, Lee JY, Nazaire F, Pushkareva M, Obeid LM, Hannun YA (1995) Role for ceramide in cell cycle arrest. J Biol Chem 270: 2047
27. Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer 26: 239
28. Kolesnick R, Golde DW (1994) The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. Cell 77: 325
29. Leonard CE, Chan DC, Chou TC, Kumar R, Bunn PA (1996) Paclitaxel enhances in vitro radiosensitivity of squamous cell carcinoma of the head and neck. Cancer Res 56: 5198
30. Levasseur LM, Greco WR, Rustum YM, Slocum HK (1997) Combined action of paclitaxel and cisplatin against wildtype and resistant human ovarian carcinoma cells. Cancer Chemother Pharmacol 40: 495
31. Liu TJ, Zhang WW, Taylor D, Roth J, Goepfert H, Clayman G (1994) Growth suppression of head and neck cancer cells by the introduction of a wild-type p53 gene via a recombinant adenovirus. Cancer Res 54: 3662
32. McGuire W, Rowinsky E, Rosenshein N, Grumbine F, Ettinger D, Armstrong D, Donehower R (1989) Taxol: a unique antineoplastic agent with significant activity in advanced ovarian epithelial neoplasms. Am J Intern Med 11: 273
33. Myrick D, Blackinton D, Klostergaard J, Kouttab N, Maizel A, Wanebo H, Mehta S (1999) Paclitaxel-induced apoptosis in Jurkat, a leukemic T cell line, is enhanced by ceramide. Leuk Res 23: 569
34. Obeid LM, Hannun YA (1995) Ceramide: a stress signal and mediator of growth suppression and apoptosis. J Cell Biochem 58: 191
35. Pulkkinen JO, Elomaa L, Joensuu H, Martikainen P, Servomaa K, Grenman R (1996) Paclitaxel-induced apoptotic changes followed by time-lapse video microscopy in cell lines established from head and neck cancer. J Cancer Res Clin Oncol 122: 214
36. Rosenthal DI, Carbone DP (1995) Taxol plus radiation for head and neck cancer. J Infus Chemother 5: 46
37. Rowinsky EK, Donehower, RC (1995) Paclitaxel. N Engl J Med 332: 1004
38. Rowinsky E, Donehower R, Jones R, Tucker R (1988) Microtubule change and cytotoxicity in leukemia cell lines treated with Taxol. Cancer Res 48: 4093
39. Schiff PB, Horwitz SB (1980) Taxol stabilizes microtubules in mouse fibroblast cells. Proc Natl Acad Sci USA 77: 1561
40. Schwartz GK, Ward D, Saltz, L, Casper ES, Spiess T, Mullen E, Woodworth J, Venuti R, Zervos P, Storniolo A, Keisen D (1997) A pilot clinical/pharmacological study of the protein kinase C-specific inhibitor saffingol alone and in combination with doxorubicin. Clin Cancer Res 3: 537
41. Sledge GW, Robert N, Goldstein LJ, et al (1993) Phase I trial of adriamycin + Taxol in metastatic breast cancer. Eur J Cancer 29A: S81
42. Smith RE, Thornton DE, Allen J (1995) A phase II trial of paclitaxel in squamous cell carcinoma of the head and neck cancer: correlative lab studies. Semin Oncol 22: 41
43. Srivastava RK, Srivastava AR, Korsmeyer SJ, Nesterova M, Cho-Chung YS, Longo DL (1998) Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cyclic AMP-dependent protein kinase. Mol Cell Biol 18: 3509
44. Steren A, Sevin B, Perras J, Angioli R, Ngugen H, Guerra L, et al (1993) Taxol sensitizes human ovarian cancer cells to radiation. Gynecol Oncol 48: 252
45. Strum JC, Small GW, Daiug SB, Daniel LW (1994) 1-β-D arabinofuranosylcytosine stimulates ceramide and diglyceride formation in HL-60 cells. J Biol Chem 269: 15493
46. Sweeney E, Sakakura C, Shirahama T, Masamune A, Ohta H, Hakomori S, Igarashi Y (1996) Sphingosine and its methylated derivative *N*,*N*-dimethyl sphingosine (DMS) induce apoptosis in a variety of human cancer cell lines. Int J Cancer 66: 358
47. Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z, Kolesnick RN (1996) Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature 380: 75
48. Villunger A, Egle A, Kos M, Hartmann BL, Geley S, Kofler R, Grell R (1997) Drug-induced apoptosis is associated with enhanced Fas (Apo-1/CD95) ligand expression but occurs independently of Fas (Apo-1/CD95) signaling in human T-acute lymphatic leukemia cells. Cancer Res 57: 3331
49. Wanebo HJ, Chougule P, Ackerley W, Konness RJ, McRae R, Nigri P, Leone L, Safran H, Webber B, Cole B (1997) Preoperative paclitaxel, carboplatin and radiation in advanced head and neck cancer (Stage III and IV) induces high rate of complete pathologic response (CR) at the primary site and high rate of organ preservation. Proc Am Soc Clin Oncol 16: 391a
50. Wang TH, Wang HS, Ichijo H, Giannakakou P, Foster JS, Fojo T, Wimalasena J (1998) Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. J Biol Chem 273: 4928
51. Westwick JK, Bielawska AE, Dbaibo G, Hannun YA, Brenner DA (1995) Ceramide activates the stress-activated protein kinases. J Biol Chem 270: 22689