Inhibition of growth and telomerase activity by novel cationic ceramide analogs with high solubility in human head and neck squamous cell carcinoma cells

MICHAEL J. ROSSI, MD KAMALA SUNDARARAJ, MS, SERAP KOYBASI, MD, MONIQUE S. PHILLIPS, BS, ZDZISLAW M. SZULC, PHD, ALICJA BIELAWSKA, PHD, TERRY A. DAY, MD, LINA M. OBEID, MD, YUSUF A. HANNUN, MD, and BESIM OGRETMEN, PHD,
Charleston, South Carolina

OBJECTIVES: Head and neck squamous cell carcinoma (HNSCC) is notoriously resistant to chemotherapy. The sphingolipid ceramide and its analogs have been demonstrated to exert antitumor activity in many cell types; however, the effectiveness of these analogs has been limited by potency and solubility. This study focuses on the effects of novel highly soluble cationic pyridinium-ceramides, alone and in combination with various chemotherapeutic agents, on cell survival, telomerase activity, and cell cycle arrest in HNSCC cell lines in vitro.

METHODS: The concentration of pyridinium-ceramides and chemotherapeutic agents that inhibited cell growth by 50% (IC_{50}) was determined by MTT cell survival assays. The cell cycle profiles were determined by flow cytometry. Telomerase activity was determined by telomerase repeat amplification protocol (TRAP) assay.

RESULTS: Treatment of the human UM-SCC-22A (SCC of the hypopharynx) cells, as well as various other HNSCC cell lines, with C_6-Pyr-Cer resulted in the inhibition of cell survival with an IC_{50} concentration of approximately 250 to 300 nM at 96 hours, whereas its IC_{50} was greater than 1000 nM in noncancerous Wi-38 human lung fibroblasts, and adult human epidermal keratinocytes. Moreover, treatment with C_6-Pyr-Cer also resulted in cell cycle arrest in G0/G1, which correlated with a significant inhibition of telomerase activity in UM-SCC-22A cells. Additional results demonstrated that the combination of C_6-Pyr-Cer with gemcitabine (GMZ) or doxorubicin (DOX), which have the lowest IC_{50} concentrations among various chemotherapeutic drugs in these cells, enhances the effects of these drugs in the inhibition of telomerase and cell growth.

CONCLUSIONS: These data suggest that the novel C_6-Pyr-Cer with high solubility and bioavailability may lead to the development of new therapeutic strategies that target telomerase for the treatment of HNSCC. (Otolaryngol Head Neck Surg 2005;132: 55-62.)

From the Department of Biochemistry and Molecular Biology (Ms Sundararaj and Phillips, and Drs Rossi, Szulc, Bielawska, Hannun, and Ogretmen), College of Medicine (Dr Rossi), Division of Head and Neck Oncologic Surgery, Department of Otolaryngology–Head and Neck Surgery (Drs Koybasi and Day), Hollings Cancer Center (Drs Szulc, Bielawska, Day, Obeid, Hannun, and Ogretmen), Medical University of South Carolina, and the Ralph H. Johnson Veterans Administration and the Department of Medicine (Dr Obeid), Charleston, SC.

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The present address for Dr Rossi is University of Pittsburgh, Department of Otolaryngology, University of Pittsburgh, School of Medicine, Pittsburgh, PA 15213.

The present address for Dr Koybasi is Abant Izzet Baysal Medical Faculty, Department of Otolaryngology, Bolu, Turkey.

Reprint requests: Besim Ogretmen, 173 Ashley Avenue, BSB 518E, Charleston, SC 29425; e-mail, ogretmen@musc.edu.

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Squamous cell carcinoma of the head and neck (HNSCC) has long been associated with poor survival and high mortality rates. Global occurrence of HNSCC is estimated to account for 5% of malignant neoplasms in the adult population. In the United States, there are approximately 40,000 cases diagnosed annually, and approximately 12,000 annual deaths.[1-3] Despite advances in treatment, including surgery, radiation, and chemotherapy, survival statistics have not improved significantly in decades.[3,4]

The sphingolipid ceramide has been shown to function as a bioeffector molecule regulating the activity of various biochemical and molecular targets. It has been shown that serine/threonine phosphatases of the PP2A and PP1 families, ceramide-activated protein phosphatases (CAPPs), are activated by ceramide in vitro.[5,6] In particular, ceramide has been shown to induce apoptosis, whereas dihydroceramide and other related lipids are inactive.[6] One of the best characterized functions of exogenous ceramide is its capability to induce a G0/G1 cell cycle arrest, which was mechanistically shown to be due to the induction of dephosphorylation of the retinoblastoma gene product (Rb).[7]

There are data that suggest that sphingolipid metabolism can be targeted to sensitize cancer cells to chemotherapy.[8] Moreover, in a recent study, it has been shown that combination of paclitaxel (PAX) and C_6-ceramide treatment synergistically decreased the number of the human Tu138 head and neck squamous carcinoma cells in the S and/or G_2-M phase of the cell cycle,[9] demonstrating a role for ceramide in the treatment of head and neck cancer cells in situ.

Previous efforts have shown that telomerase, an RNA-dependent DNA polymerase that elongates telomeres at the end of chromosomes, is active in approximately 80% to 90% of the tumor tissues of the HNSCCs, whereas it is not active in normal head and neck tissues.[10] Because abnormal activation of telomerase in tumors appears to be required for their immortality, these data suggest telomerase as a significant therapeutic target for the development of cancer-specific novel treatment strategies of HNSCCs. In this regard, recent studies in our laboratory have shown that ceramide mediates the inhibition of telomerase activity in the A549 human lung adenocarcinoma cells.[11] The results showed that the inhibition of telomerase can be induced in response to exogenous C_6-ceramide and endogenous long chain ceramides,[12] and that this inhibition is independent of apoptosis, but correlated with G0/G1 cell cycle arrest in these cells.[11] Further data have shown that the regulation of telomerase by ceramide involves the inactivation of c-Myc transcription factor via increased ubiquitin/proteasome function for its rapid proteolysis.[13] These results therefore demonstrate that ceramide is a candidate upstream regulator of telomerase. In this study, we examined the roles of newly developed water-soluble C_6-Pyr-Cer in the inhibition of cell cycle and telomerase activity as compared with the conventional ceramides and/or chemotherapeutic agents, alone or in combination, in various HNSCC cell lines.

MATERIALS AND METHODS

Ceramides and Chemotherapeutic Agents

Short chain ceramide analogs (D-erythro and L-threo-ceramides with C_2-C_{16} fatty acid chain lengths) were obtained from the Synthetic Lipidomics Core at the Department of Biochemistry and Molecular Biology at MUSC. The novel water soluble pyridinium salt-conjugated ceramides were synthesized by the Lipid Chemistry and Cell Biology Laboratory at the Department of Biochemistry, Medical University of South Carolina as described (Szulc Z. et al., unpublished data). Cetylpyridinium bromide (CPB) monohydrate was purchased from Aldrich.

Cell Lines and Culture Conditions

Human head and neck cancer cell lines[14] UM-SCC-1 (retromolar trigone/floor of the mouth), UM-SCC-12 (primary tumor of larynx), and UM-SCC-22A cells were obtained from Dr. Thomas Carey at the Department of Otolaryngology—Head and Neck Surgery, University of Michigan. Cells were grown in Dubecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin at 37ºC in 5% CO_2. The Wi-38 human lung fibroblasts were obtained from American Type Culture Collection, and were grown in DMEM as described above. Adult human epidermal keratinocytes were purchased from Cascade Biologics, and were maintained in EpiLife medium with growth supplements as described by the manufacturer.

MTT Cell Survival Assay and Isobologram Studies

The concentrations of agents that inhibited cell growth by 50% (IC_{50}) were determined from cell survival plots obtained by MTT assay as described.[11] Triplicate wells were used for each treatment. The final concentration of DMSO [a solvent for conventional ceramides, gemcitabine (GMZ) and doxorubicin (DOX)] in the growth medium was less than 0.1% (v/v), which has no effect on cell growth and survival. Pyridinium ceramides were dissolved in sterile water or growth medium. In short, after cells (5-10 x 10^3/well) were seeded in 96-well plates for 24 hours, they were treated with increasing concentrations of ceramide analogs for an additional 48 or 96 hours. For combination treatments, ceramides and chemotherapeutic agents (GMZ or DOX) were added to the growth medium simultaneously during treatment. Isobologram plots were then constructed using IC_{50} values of the 2 agents alone or in combination obtained from MTT assays. A straight line joining points on x- and y-axes represent the IC_{50} concentrations of the 2 agents, and the points representing the IC_{50} concentrations of the combination of the 2 agents are represented as scatter plots on the same graphs. In general, the points that fall within the left of the straight line indicate synergism. The experiments were performed as triplicates in at least 3 independent experiments.

Analysis of Cell Cycle Profiles

The effects of pyridinium ceramides on the cell cycle profiles of A549 cells at 24 hours were analyzed in the presence of DNase-free RNase and propidium iodine (PI) by flow cytometry as described previously.[11] Untreated cells were used as controls.

Determination of Telomerase Activity

Telomerase activity in cell extracts was measured by the polymerase chain reaction-based telomere repeat am-

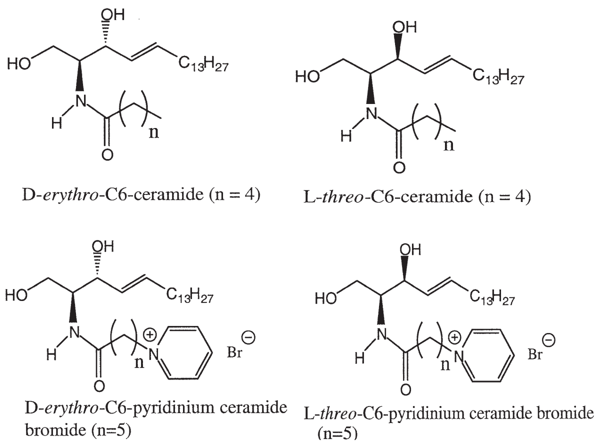

Fig 1. Chemical structures of the conventional and novel cationic ceramides. The chemical structures of conventional ceramides (D-e- and L-th-C_6-ceramides) and newly developed cationic pyridinium-ceramides (D-e- and L-th-C_6-Pyr-Cers) are shown.

plification protocol (TRAP) using TRAPeze kit (Intergen, Gaithersburg, MD), which includes a 36-bp internal control to allow quantification of activity as described.[11-13] In short, the intensity of telomere-specific DNA bands, measured using Quantity One (BioRad) software, were normalized to the intensity of internal control bands for each sample on polyacrylamide gels for quantification.

RESULTS

Exogenous ceramides have been shown to mediate antiproliferative responses such as cell cycle arrest, apoptosis, or senescence in various cancer cells. However, because the solubility and bioavailability of these exogenous ceramides are known to be limited, the water-soluble pyridinium-conjugated analogues of ceramides were developed. Chemical structures of conventional D-erythro (D-e)- and L-threo (L-th)-ceramides and their novel cationic analogs are shown in Figure 1.

First, to examine the effects of pyridinium ceramides containing different fatty acid chain length in cell growth, the cells were treated with C_6-, C_{12}-, and C_{16}-D-e- and L-th-pyr-cer for 48 hours (Fig 2A and B, respectively). The IC_{50} concentrations of C_6-, C_{12}- and C_{16}-D-e-, and L-th-Pyr-Cer were comparable in UM-SCC-22A cells at 48 hours of treatment (Fig 2A and B), and these values (approximately 1 μM) were higher than those obtained after 96-hour treatment (approximately 300 nM). These results also demonstrate that the longer fatty acid chains in these pyridinium-conjugated ceramide analogs did not appear to play a major role in their growth suppressing effects in these cells. Because the IC_{50} concentrations of D-e- and L-th-C_6-Pyr-Cer were almost identical (at 48 and 96 hours), they have been used interchangeably throughout this study. The effects of C_6-Pyr-Cer on the inhibition of cell survival were also examined in other HNSCC cell lines (UM-SCC-1 and UM-SCC12), and similar results with IC_{50} values between 250 and 300 nM at 96 hours were obtained (data not shown).

Next, the growth inhibitory effects of conventional exogenous ceramides, D-erythro (D-e-)-C_6-ceramide and L-thereo (L-th)-C_6-ceramide, and their newly developed highly water-soluble cationic analogs, D-e-C_6-Pyr-Cer and L-th-C_6-Pyr-Cer in the UM-SCC-22A cells were determined using MTT assays after 96 hours of treatment. As seen in Figure 2C, D-e-C_6-Pyr-Cer and L-th-C_6-Pyr-Cer showed IC_{50} values ranging from 250 to 300 nM, whereas conventional C_6-ceramides failed to achieve an IC_{50} at concentrations up to 1300 nM, demonstrating that the newly developed C_6-Pyr-Cer is greater than 4-fold more effective in inhibiting cell growth. Interestingly, it seems that C_6-Pyr-Cer contains the optimum chain length for growth inhibition, because the C_2-Pyr-Cer did not exert a significant effect on cell growth (Fig 2C). Thus, to date, these novel C6-Pyr-ceramides represent the most potent analogs of ceramide in exerting growth-suppressing activity.

To evaluate the effects of the Lt-C_6-Pyr-Cer in noncancerous cells, the inhibition of cell growth in noncancerous Wi-38 human lung fibroblasts and adult human epidermal keratinocytes (HEK) in response to increasing concentrations of C_6-pyridinium ceramide was assessed. Because the contents of growth media to maintain HEK and UM-SCC-22A cells in culture are largely different than each other, the effects of C_6-Pyr-Cer on the growth of HEK and UM-SCC-22A cells were examined in 2 independent experiments by growing both of these cell lines either in the DMEM containing 10% FCS, or in EpiLife medium, with growth supplements in which HEK cells are normally maintained. Interestingly, C_6-Pyr-Cer was not active in EpiLife medium, IC_{50} concentrations were greater than 2 μM at 96 hours for both of the cell lines. However, when these cell lines were grown in DMEM, the IC_{50} value of C_6-Pyr-Cer was greater than 1000 nM (Fig 2D) for keratinocytes, whereas its IC_{50} concentration in UM-SCC-22A cells was approximately 300 nM. Similar results were also obtained using Wi-38 fibroblasts (grown in DMEM), in which IC_{50} concentration of C_6-Pyr-Cer was greater than 1000 nM (Fig 2D). These results, therefore, suggest that C_6-Pyr-Cer is greater than 4-fold more effective in inhibiting cell growth in squamous cell carcinoma cells than noncancerous fibroblasts or keratinocytes in vitro, indicating a lower potential for general toxicity. This was further confirmed in vivo, in which the maximum tolerated dose

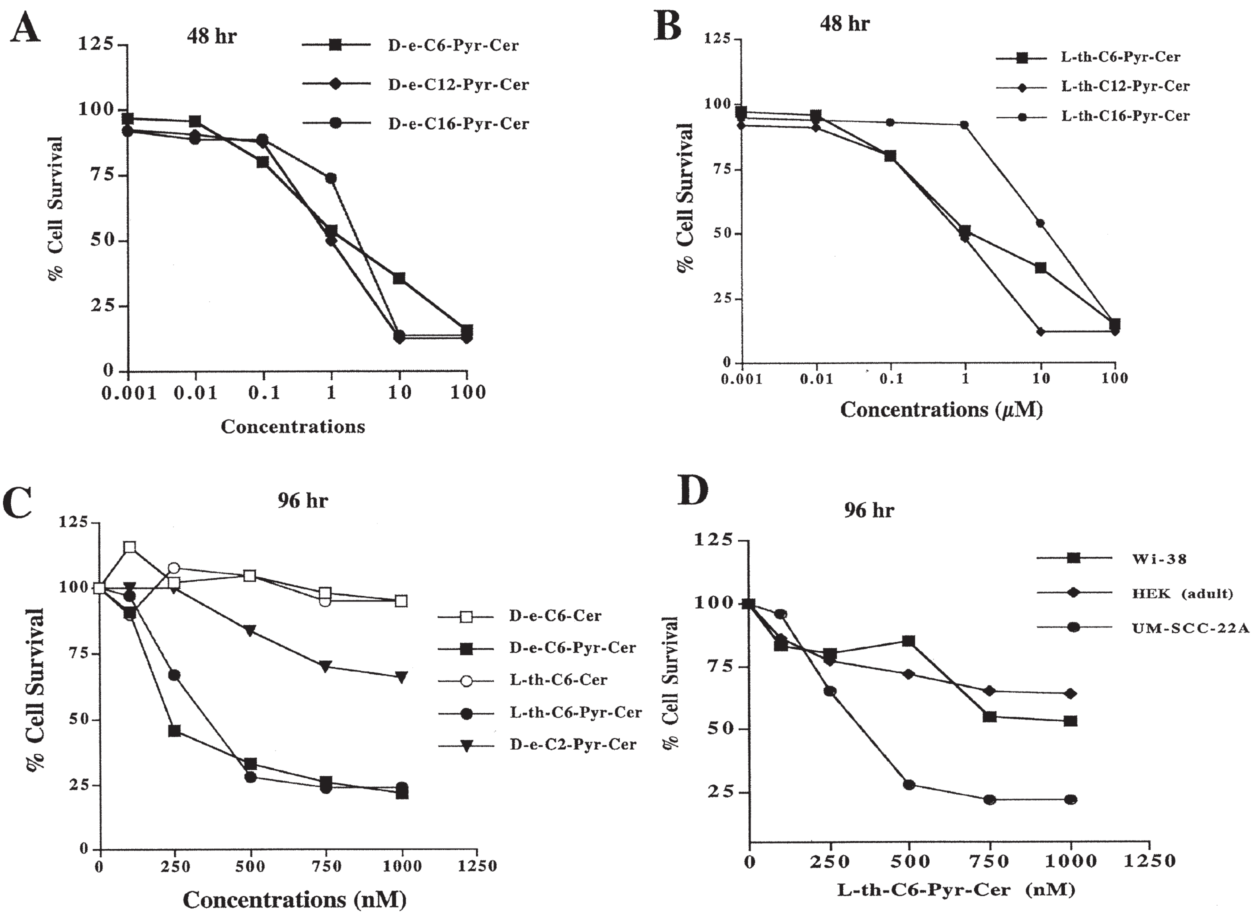

Fig 2. Effects of pyridinium ceramides on cell survival in vitro. The IC_{50} concentrations of D-e- and L-th-C_6-, C_{12}-, and C_{16}-pyridinium-ceramides were determined by MTT assays (**A** and **B**, respectively).The effects short chain pyridinium ceramides on cell growth were determined and compared to that of conventional ceramides using MTT assays (**C**). The effects of L-th-C_6-ceramide on cell growth in UM-SCC-22A, noncancerous Wi-38, and HEKa cells were also examined using MTT assays as described in "Materials and Methods" (**D**). The MTT assays were performed using triplicate samples in at least 2 independent experiments. Standard deviations for each point were between 0.5% and 5%.

(MTD) of C_6-Pyr-Cer with no detectable toxicity in SCID mice was found to be around 80-100 mg/kg, which is comparable to the MTD of GMZ (80 mg/kg) whereas the MTD of DOX was between 1-2 mg/kg in mice (Senkal CE, Rossi MJ, Ogretmen B, unpublished data).

It has been shown previously that ceramide treatment results in cell cycle arrest in G0/G1 of many cell types. Therefore, the effects of pyridinium-ceramides on cell cycle profiles and telomerase activity in UM-SCC-22A cells were examined using flow cytometry and TRAP assays as described in "Materials and Methods." The results showed that treatment of UM-SCC-22A cells with D-e-C_6-, C_{12}-, and C_{16}-Pyr-Cer at 1 μM for 48 hours resulted in a significant increase in the population of cells in G0/G1 when compared to untreated controls (Fig 3A). Thus, these analogs caused an arrest in cycle progression.

Importantly, examination of apoptotic cell death using annexin V staining in response to C_6-Pyr-Cer did not show any detectable signal (data not shown), and no sub G0/G1 apoptotic peaks in flow cytometry analysis were observed in the presence or absence of C_6-Pyr-Cer in these cells (Fig 3A). Thus, these ceramide analogs appear to exert minimal apoptotic activity on their own, with selective cell cycle arrest in these cells.

The selective effects on cell cycle raised the possibility that these compounds may regulate telomerase function. Indeed, treatment of cells with 100, 250, and 500 nM C_6-Pyr-Cer for 96 hours causes approximately 30, 50, and 75% inhibition of telomerase activity, respectively, when compared with that of untreated controls (Fig 3B, lanes 2−4 versus 1). Additional results also showed that C_6-Pyr-Cer had no significant effect on telomerase activity when applied directly to the extracts in vitro even at 10, 20, and 100 μM (Fig 3B,

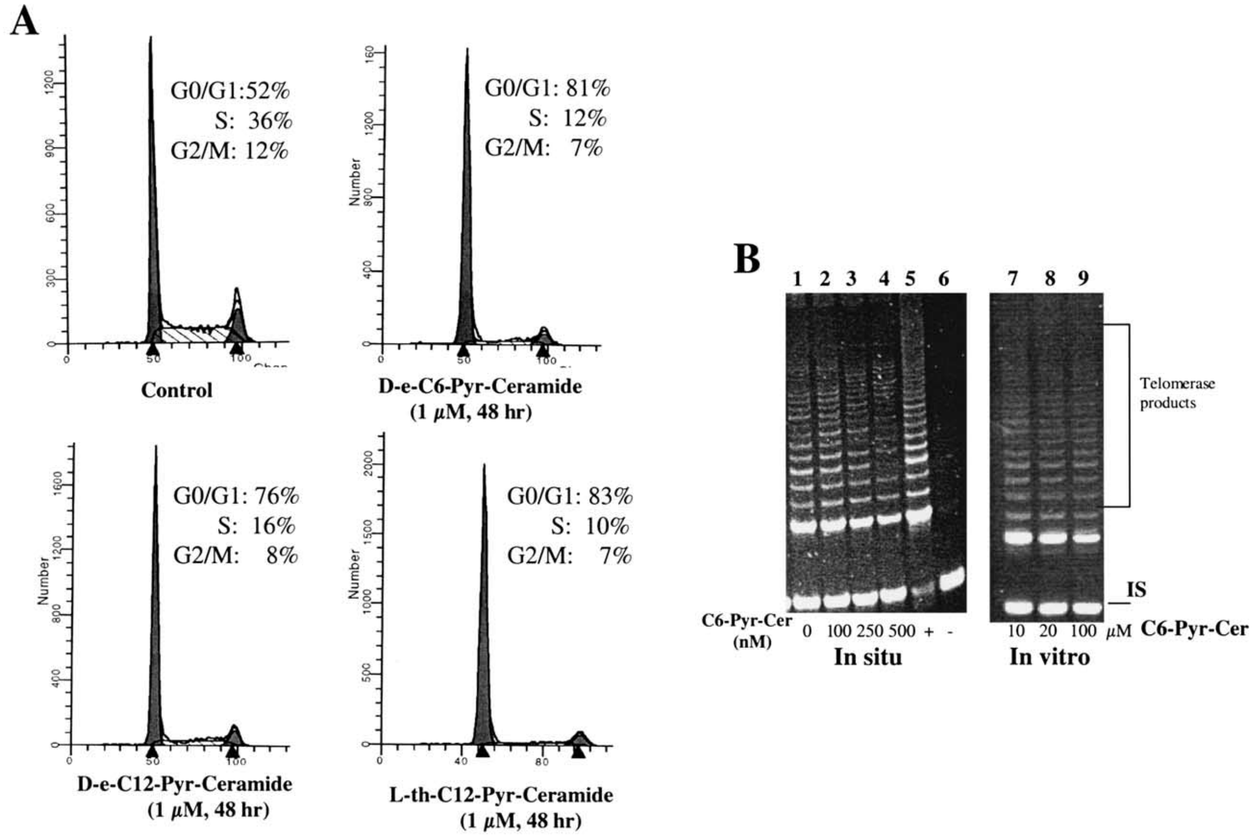

Fig 3. Roles of C_6-Pyr-Cer on cell cycle profiles, telomerase activity, and telomere length of UM-SCC-22A cells. (**A**) The effects of D-e-C_6-, C_{12}-, and C_{16}-pyridnium ceramides (at 1 μM for 48 hours) on cell cycle profiles of UM-SCC-22A cells were determined and compared to that of untreated cells using flow cytometry as described in "Materials and Methods." (**B**) The effects of 0, 100, 250, and 500 nM L-th-C_6-Pyr-Cer (lanes 1−4, respectively) at 96-hour treatment in UM-SCC-22A cells on telomerase activity was assessed using TRAP assay. Lanes 5 and 6 contain positive (+) and negative (−) controls. To examine whether C_6-Pyr-Cer inhibits telomerase activity directly, cell extracts (as shown in lane 1) were incubated in the presence of various concentrations of L-th-C_6-ceramide (10, 20, and 100 μM) and telomerase activity was determined using TRAP assay (lanes 7−9, respectively). The presence of nontelomeric 36-bp internal standard (IS) in the TRAP assay serves as a control for polymerase chain reaction, and used for quantitation/normalization of. the activity of telomerase. The results shown are representative of at least 2 independent experiments.

lanes 7−9), demonstrating that the inhibition of telomerase by C_6-Pyr-Cer is not due to a direct inhibition of the enzyme, but involves transcriptional and/or post-transcriptional regulation of telomerase. Thus, these data demonstrate that these ceramide analogs cause a significant inhibition of telomerase, which correlates with cell cycle arrest at G0/G1 in UM-SCC-22A cells.

Clinical data suggest that head and neck cancers respond better to chemotherapy regimens that include combinations of 2 or more anticancer agents.[15] Indeed, the conventional chemotherapy for these cancers is the combination of cisplatinum (CSP) with 5-fluorouracil (5-FU), or taxol.[15] Therefore, experiments were conducted to determine whether the combination of C_6-Pyr-Cer would be synergistic in inhibiting growth when combined with anticancer drugs. First, the IC_{50} values of various chemotherapeutic agents were determined in UM-SCC-22A cells using MTT assays. As shown in Figure 4A, DOX, daunorubicin (DNR), methotrexate (MTX), and GMZ exhibited the lowest IC_{50} values between 100 and 250 nM, whereas CSP, PAX, carboplatinum (CRBP), and 5-FU showed IC_{50} values between 1 and 100 μM in these cells.

Next, experiments were performed to determine whether C_6-Pyr-Cer inhibited cell growth synergistically in combination with DOX in UM-SCC-22A cells, the most potent inhibitor of cell survival in these cells. Quantitative isobologram studies were performed as described in "Materials and Methods." The results demonstrated that the combination of C_6-Pyr-Cer at 25, 50, and 100 nM with DOX for 96 hours decreased growth synergistically, detected as shift of the IC_{50}

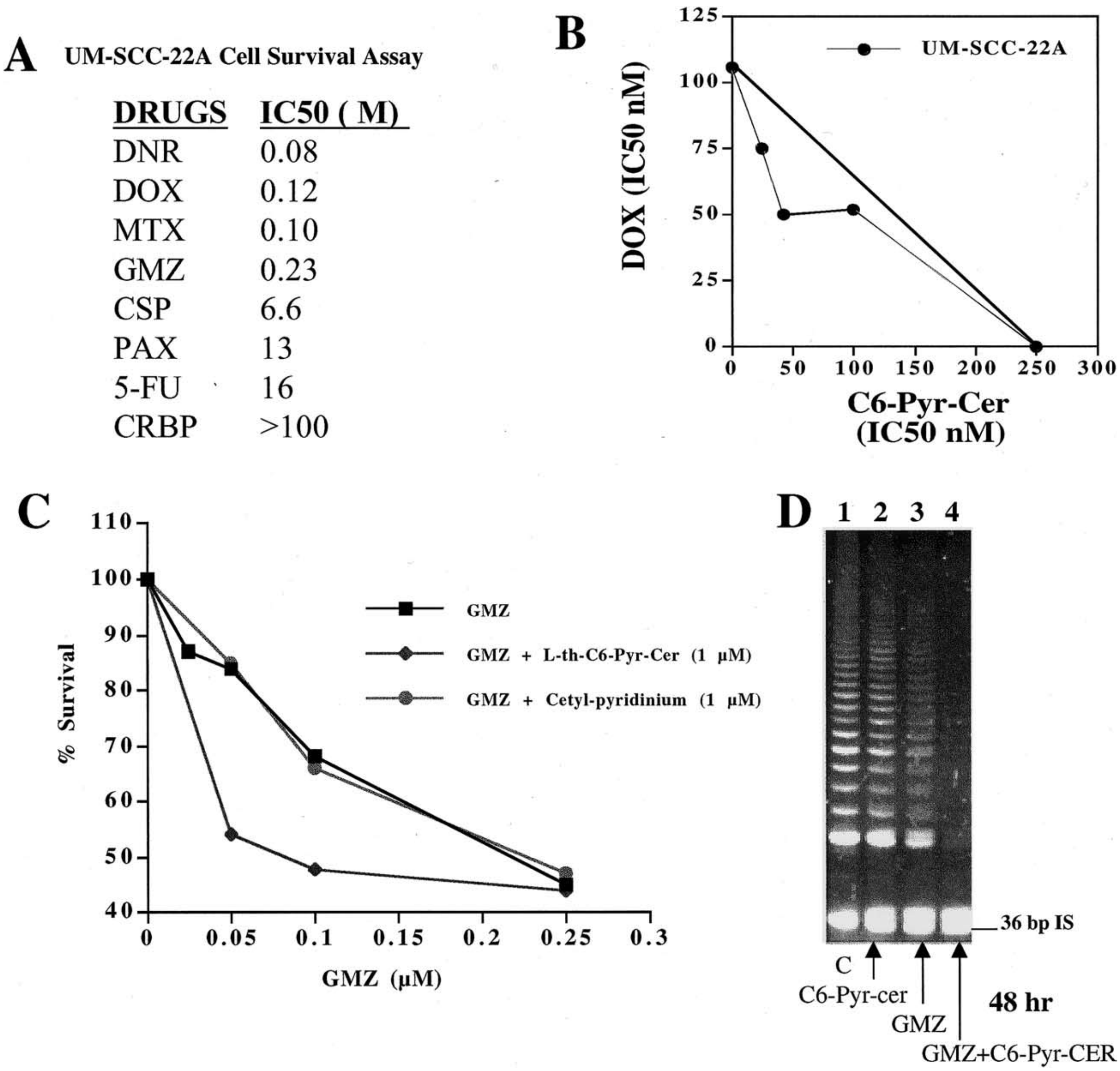

Fig 4. Synergistic effects of C_6-Pyr-Cer in combination with GMZ or DOX on cell growth and telomerase activity in UM-SCC-22A cells. (**A**) The effects of various chemotherapeutic agents on cell growth in UM-SCC-22A cells were determined using MTT assays, and the IC_{50} concentrations of each agent were determined from cell growth plots as described. (**B**) The synergistic effects of DOX and L-th-C_6-Pyr-Cer on cell growth were assessed using isobologram studies as described in "Materials and Methods." (**C**) The effects of 1 µM L-th-C_6-Pyr-Cer or cetyl-pyridinium-bromide, without ceramide conjugate (used as a control) on the growth inhibition potential of GMZ in UM-SCC-22A cells were determined using MTT assays. (**D**) The effects of GMZ (100 nM), L-th-C_6-Pyr-Cer (1 µM), or GMZ and L-th-C_6-Pyr-Cer (100 nM and 1 µM, respectively) on telomerase activity in UM-SCC-22A cells at 48 hours were examined using TRAP assay, and compared to that of untreated controls (lanes 2, 3, 4, and 1, respectively). The data shown are representative of at least 2 independent experiments performed in triplicate samples. Standard deviations in each sample were between 0.1 and 0.5.

values of DOX in the isobologram (Fig 4B) to the left of the line plot joining the x- and y-axes that represent the IC_{50} of C_6-Pyr-Cer and DOX alone, respectively. Interestingly, the optimum concentration of C6-Pyr-Cer was 50 nM for its synergistic interaction with DOX, and using 100 nM C6-Pyr-Cer did not enhance synergy with DOX in these cells (Fig 4B).

Because GMZ has been reported to delay tumor development in a xenograft model of human squamous cell carcinoma-contaminated surgical wounds,[16] and it was also found to be 1 of the more potent inhibitors of cell growth in UM-SCC-22A cells in the present study, the effects of treatment of cells with the combination of C_6-Pyr-Cer on cell survival and telomerase activity were assessed. As seen in Figure 4C, combining 1 μM C_6-Pyr-Cer with various concentrations of GMZ for 48 hours of treatment enhanced the antiproliferative effects of GMZ when compared with treatments with GMZ alone. The combination of cetyl-pyridinium itself without conjugation to ceramide (used as a nonspecific control) with GMZ did not have any role on the efficacy of GMZ in these cells (Fig 4C), demonstrating a specific role for ceramide in the synergistic growth inhibition response to GMZ in these cells. To determine whether C_6-Pyr-Cer and GMZ also synergistically inhibited telomerase activity, UM-SCC-22A cells were treated with C_6-Pyr-Cer (1 μM), GMZ (100 nM), or the combination of C_6-Pyr-Cer and GMZ for 48 hours. Then, telomerase activity was measured using TRAP assay as described in "Materials and Methods." As seen in Figure 4D, treatment of UM-SCC-22A cells with C_6-Pyr-Cer (lane 2) or GMZ (lane 3) resulted in the 30% to 50% inhibition of telomerase activity, respectively, whereas treatment of cells with the combination of C_6-Pyr-Cer and GMZ (lane 4) caused a significant additional inhibition of telomerase activity (approximately 90%) in these cells, as compared with untreated controls (lane 1).

Taken together, these results suggest that these newly developed cationic pyridinium ceramides can synergistically inhibit cell survival when combined with chemotherapeutic drugs for the treatment of HNSCC.

DISCUSSION

The results presented herein demonstrate that the newly developed novel water-soluble ceramide analogs (pyridinium-ceramides) can inhibit cell growth of HNSCCs, with approximately 250 to 300 nM IC_{50} concentrations in vitro. The data demonstrate that the action of C_6-Pyr-Cer mechanistically involves the inhibition of telomerase, which correlates with growth arrest at G0/G1 in these cells. In addition, the treatment of noncancerous Wi-38 or adult HEK cells, which do not express detectable telomerase activity, with C_6-Pyr-Cer did not cause a significant inhibition of cell survival, indicating a lower potential for overall toxicity. More importantly, it has been also demonstrated in this study that pyridinium-ceramides can potentiate the antiproliferative effects of known chemotherapeutic drugs such as GMZ and DOX in UM-SCC-22A cells.

The standard of treatment for advanced HNSCC is continuing to evolve, with increased emphasis on the use of organ-sparing chemoradiotherapy regimens.[17] Although survival data are comparable for early stage T1−T2 oral cavity tumors with either radiation therapy or surgery, most oral cavity cancers are treated surgically.[18] Postoperative radiation therapy may be beneficial for stage III and IV oral cancer, although chemotherapy has not been proven as a curative option for oral cancer at this time.[19] Surgical resection requires complete tumor removal and frozen section margin analysis remains the standard of care. Novel molecular diagnostic techniques are currently being studied as markers for residual, persistent, and recurrent disease. Recent studies have shown that telomerase is activated in approximately 90% of the HNSCC tumors, although it is not active in the majority of normal head and neck tissue,[10] demonstrating that the inhibition of telomerase might provide cancer-specific therapeutic strategies for the treatment of HNSCC. The results here show that 1 of the mechanisms involved in the inhibition of cell survival by pyridinium-ceramides is the inhibition of telomerase. We have shown previously that ceramide is an upstream regulator of telomerase, and the inhibition of telomerase by ceramide is mainly linked to rapid degradation of c-Myc via the ubiquitin/proteasome pathway.[12] However, the molecular mechanisms by which pyridinium-ceramides regulate the activity of telomerase are not known, and need to be determined.

The conventional chemotherapy of the HNSCCs in the clinic involves mainly the combination of CSP with 5-FU or PAX.[15] However, these compounds had no significant effects on cell growth when applied either alone or in combination (data not shown) in UM-SCC-22A cells. Interestingly, GMZ and DOX seem to be very effective in inhibiting the growth of these cells in vitro, with very low IC_{50} concentrations. Recent studies have evaluated the combination of GMZ with the antineoplastic vinka alkaloid vinorelbine in HNSCCs, which showed no reported pharmacokinetic or synergistic interaction.[20] Combination therapy involving anthracyclines has been analyzed recently, advocating the dosage limitations of these cardiotoxic compounds in HNSCCs.[21] However, because the results with GMZ in combination with pyridinium ceramides appear to be particularly promising, their synergistic roles in the inhibition of cell growth in HNSCC need to be evaluated in vivo using animal models and phase I clinical trials.

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