Complete Inhibition of Growth followed by Death of Human Malignant Melanoma Cells in Vitro and Regression of Human Melanoma Xenografts in Immunodeficient Mice Induced by Camptothecins

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ABSTRACT

The plant alkaloid camptothecin and three camptothecin derivatives were used to study responses of human malignant melanoma (BRO) cells xenografted in immunodeficient (nude) mice. Camptothecin and its derivatives 9-nitro-20(S)-camptothecin and 9-amino-20(S)-camptothecin inhibited growth of tumors and caused regression in all tumor-bearing mice. Tumor regression was accompanied by degenerative changes in the tumor cells, as assessed by microscopic observations of histological sections prepared from the tumors. No toxic effects were observed in the drug-treated mice, with or without xenografts. In parallel experiments, camptothecin, 9-nitro-20(S)-camptothecin, and 9-amino-20(S)-camptothecin inhibited proliferation of BRO cells in vitro and resulted in dramatic morphological cellular changes comparable to those observed in the sections of solid tumors. The derivative 12-nitro-20(S)-camptothecin had no effect on BRO tumors or cell cultures. The difference between 9-nitro-20(S)-camptothecin and 12-nitro-20(S)-camptothecin is the position at which the NO2 group is attached to the camptothecin molecule. In contrast to BRO melanoma cells, none of the camptothecin derivatives had any effect on cultured human melanocytes, the normal counterparts of melanoma cells. Taken together, the findings indicate that camptothecin and derivatives exert different effects on the growth and morphology of normal and malignant (BRO) melanocytes.

INTRODUCTION

The plant alkaloid CAM is a potent inhibitor of cellular RNA and DNA synthesis but not of protein synthesis (1). Studies with isolated DNA and purified topoisomerase I showed that both inhibition of topoisomerase I-mediated DNA relaxation and induction of DNA nicks by CAM begin at approximately 0.35 μg/ml and reach the 50% point at about 3.5 μg/ml CAM (2). Further, CAM induces DNA strand breaks in intact murine L1210 cells and isolated nuclei between 0.15 and 0.35 μg/ml (3). CAM is cytotoxic for L929 murine fibrosarcoma and ME-180 human cervical carcinoma cell lines, with 50% lethal doses of 17.5 μg/ml and 1.75 μg/ml, respectively (4). Flow cytometry studies showed that CAM-treated human leukemia cells exhibit DNA degradation in S-phase or are arrested in S- and G2-phases, depending on whether the leukemia cells are of myeloid or lymphoid lineage (5, 6). Also, we have shown recently that CAM, 9NC, and 9AC induce expression of the c-jun and jun-B genes in U937 human leukemia cells and that induction of these genes is associated with internucleosomal DNA cleavage (7). It is now documented that CAM does not bind DNA or topoisomerase I alone but interferes with the breakage-reunion process of topoisomerase I by stabilizing the enzyme-DNA “cleavable complexes,” an event which leads to fragmentation of nuclear DNA (8). The sensitivity of malignant cells to CAM has been correlated positively with the amount of topoisomerase I possessed by these cells (9-11). While CAM and its derivatives are known inhibitors of topoisomerase I, the basis for their selective antitumor activity is unclear.

Water-soluble sodium CAM was used to treat cancer patients in clinical trials in the 1970s (12-15), but high toxicities and low antitumor activity led to its discontinuation. Recently, a derivative of CAM, camptothecin-11, has been used in phase II clinical studies on patients with refractory leukemias and lymphomas, with some effectiveness (16). Also, camptothecin-11 has been used in phase I and II studies on patients with advanced non-small cell lung carcinoma (17) and gynecological cancers (18), respectively, but the response rate was low.

We report here effects of CAM and CAM-derivatives on human malignant melanoma cells (BRO cell line) and normal melanocytes in vivo and in vitro. The results indicate that malignant BRO cells respond to the treatments similarly both in vivo and in vitro. Also, the results suggest that CAM and CAM derivatives, if appropriately administered, may effectively inhibit progression and induce regression of malignant melanoma.

MATERIALS AND METHODS

Drugs. Semipurified CAM was obtained from Sigma (St. Louis, MO) and the Institute of Materia Medica, Academia Sinica (Shanghai, China) and further purified in our laboratory. The CAM derivatives 9AC, 9NC, and 12NC were synthesized in our laboratory according to a published procedure (19). Purified CAM, 9NC, 9AC, and 12NC exhibited single peaks when analyzed by high performance liquid chromatography. Purified CAM and its derivatives were stored in desiccators under N2 at -70°C. For the in vivo studies, the drugs were used as a fine suspension in Intralipid 20% (KabiVitrum, Inc., Franklin, OH) and injected into mice i.m. in the muscles of the posterior legs. For the in vitro studies, the drugs were prepared as a fine suspension in 10% EG 400 (Aldrich, Milwaukee, WI), at a concentration of 5 μg/ml, and were stored at -70°C until used.

Cells and Cultures. BRO melanoma cells were derived from a biopsy of a human primary melanoma of the skin and then transplanted in nude mice, where they induce highly malignant tumors without a characteristic pattern of metastasis (20). The cells at passages 20-30 were grown in RPMI medium supplemented with 10% fetal calf serum and the antibiotics penicillin and streptomycin. PEG and PEG suspensions of CAM or derivatives were added to cell culture flasks under gentle agitation. Cell cultures were grown in T25 Falcon flasks (Becton-Dickinson, Oxnard, CA) at 37°C in a humidified CO2 incubator. After desired periods of treatment, the cells were stained for microscopy or harvested by trypsinization and counted. The normal counterparts of BRO melanoma cells, human epidermal melanocytes, were obtained...
from Clonetics Corporation (San Diego, CA), grown in melanocyte growth medium, and used at passages 4–8.

Nude Mice and BRO Xenografts. In the present studies we used 80 ± 5-day-old male Swiss nude mice of the NIH-1 high fertility strain, routinely bred and maintained in a pathogen-free environment (21). Approximately \(10^7\) BRO cells were inoculated per mouse at day 0, as described (20). To study inhibition of tumor growth, injections with CAM derivatives were initiated 1–9 days following cell inoculation. To study regression of melanomas, drug treatment was initiated when the tumor reached the desired size. Drugs were injected i.m. twice weekly at doses of 4 mg/kg of body weight. Tumor growth inhibition was monitored by measuring the tumor size of treated and untreated animals. Drug toxicity was monitored by measuring body weight of mice with and without tumor xenografts.

Preparation of Samples for Microscopy. BRO tumors were removed surgically or with the aid of a 2-mm biopsy punch (Baker-Cummins Pharmaceuticals, Inc., Miami, FL), and histological sections were prepared and stained with hematoxylin as described (20). Control and drug-treated cultures were fixed on T25 culture flasks (Falcon) and stained with methylene blue, as described (22). The flasks were then cut with a hot platinum wire, and dried preparations of stained cells were covered with immersion oil (type A; Cargille Laboratories, Cedar Grove, NJ) and a coverglass. Slides of histological sections and flasks of fixed-stained cells were examined under Nikon and Zeiss microscopes. Photomicrographs were taken on Gold 100 Kodak film.

RESULTS

Inhibition by Camptothecins of Growth of Melanoma Tumors in Vivo. In this study, \(10^7\) BRO cells were inoculated into the mice as described (20), and the first drug treatment was administered 7 days later. Measurements of tumor size and body weight started 2 days after the first drug treatment. Six mice were treated with CAM, six with 9AC, six with 9NC, and six with 20% Intralipid only. The measurements of tumor growth are shown in Fig. 1. The growth rate of tumors varied somewhat from mouse to mouse, but the pattern was similar in all inoculated mice. Of the control mice, one died at day 48 with a tumor size of 48,000 mm\(^3\), two at day 61 with respective tumor sizes of 65,000 mm\(^3\) and 105,000 mm\(^3\), and one at day 72 with a tumor size of 78,000 mm\(^3\). In contrast, all mice that received treatments with CAM, 9AC, or 9NC were virtually tumor-free by the time all controls had died. The inhibitory effect of CAM, 9NC, and 9AC on the growth of BRO xenografts is demonstrated in Fig. 1, A, B, C, and D. In this experiment, all drug-treated mice were virtually free of tumor after a treatment period of about 40 days, while all untreated mice exhibited enormous tumors. The dramatic effects of 9NC administration are even more emphatically demonstrated in the experiment shown in Fig. 2. Small size tumors were seen in both groups of animals 9 days after the animals had received BRO xenografts (Fig. 2, A and B). However, there was a dramatic difference between the untreated mice and those that received 9NC for 33 days. 9NC-treated mice were free of tumors and very active (Fig. 2D), whereas untreated mice all carried oversize and ulcerated tumors (Fig. 2C). During the period of drug treatment, we also monitored changes in body weight, an indicator of extent of toxicity of drugs in treated mice. The dramatic increase in body weight seen in untreated mice was caused by the growing tumors (Fig. 3A). In contrast, all drug-treated mice exhibited only small changes in body weight throughout the experiment. The small initial weight loss is due to the disappearance of the xenograft (Fig. 3B, C, and D).

Regression of Human Melanoma Tumors. We further studied the effectiveness of CAM and CAM derivatives on melanoma tumors already established in mice. For this study, mice were inoculated with BRO melanoma cells, and treatments were started when tumors reached the desired sizes, which varied from 100 to 1000 mm\(^3\). The effect of 9NC on the tumor size is shown in Fig. 4. This effect was immediate in palpable tumors, inasmuch as regression could be observed within 7 days of the first drug treatment (Fig. 4B). Tumors of 0.5 cm\(^3\) in size started regressing after about 2 weeks of drug treatment. A longer period of 9NC treatment, of about 30 days, was required in order to observe regression in tumors of 1-cm\(^3\) size. Tumor-bearing mice that did not receive drug treatment were dead by the completion of this experiment period, after they had developed oversize tumors (Fig. 4A). Measurements of body weight (Fig. 5) showed a dramatic increase in these untreated mice (Fig. 5C), which paralleled the dramatic increase in tumor size (Fig. 4A). Tumor-bearing mice exhibited a small weight loss at the beginning of treatment, caused by tumor regression, with no significant changes observed afterwards (Fig. 5, D, E, and F). We also measured the weight of mice that neither were inoculated with any xenografts nor received drug treatment. Their weight remained essentially unchanged throughout the experiment (Fig. 5A). Finally, to investigate toxic effect(s) of 9NC, we monitored weight changes in mice that did not receive xenografts but were treated with 9NC. The results indicate that 9NC-treated mice maintained a rather stable body weight (Fig. 5B), like the mice in Fig. 5A.

In all mice, but particularly in those bearing 1-cm\(^3\) tumors, tumors did not appear to be eliminated completely, even after prolonged treatment with 9NC for about 150 days. This was not due to malignant cells resisting 9NC, but rather, to accumulation of nonproliferating normal stromal cells, residue of the tumor stroma, which is not affected by the action of 9NC.
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Fig. 2. Inhibition of growth of human BRO melanoma xenografts. In this experiment, nude mice were inoculated with BRO cells and then randomly divided into two groups. Nine days later, one group of mice received no drug treatment (A), whereas the other group (B) received the first of the 9NC treatments, which were administered twice a week. Photographs were taken at that time. Forty-two days after cell inoculation, the untreated (C) and 9NC-treated (D) mice were rephotographed.

"Pathology of Tumors" and "Growth Inhibition of Melanoma Cells in Vitro".

Pathology of Tumors. Regression of tumors produced by 9NC treatment was also studied by microscopy of histological sections of tumors excised from untreated and 9NC-treated mice. Microphotographs of these sections are shown in Fig. 6. Fig. 6a shows a typical melanoma tumor with several mitotic cells. Fig. 6b shows a section from a tumor treated with 9NC for 3 days. The melanoma cells exhibit an increase in overall size and in nuclear size. Many of the cells possess two or more nuclei. A major characteristic in the section shown in Fig. 6b is the presence of large intercellular spaces among the enlarged melanoma cells. Longer treatment with 9NC resulted in more enlarged melanoma cells with a "flattened" appearance. Many cells possessed several nuclei at this point. Fig. 6c shows a section prepared from a tumor treated with 9NC for 14 days. Less intercellular space is visible in Fig. 6c than in Fig. 6b, apparently because of the presence of many very enlarged melanoma cells. Viable stromal cells became more and more prominent in the sections with the progressive disappearance of the tumor cells. When no further gross tumor regression was observed in 9NC-treated mice, histological studies showed that...
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Fig. 4. Regression of melanoma tumors induced by 9NC. Nude mice with BRO tumors of various sizes were treated with 9NC, and tumor size was monitored throughout the treatment period. Mean values, extreme values, and surviving animals are reported as in Fig. 1. Mice inoculated with BRO cells did not receive drug treatment (A) or received 9NC treatment when the tumor size was palpable (B), 0.5 cm³ (C), or 1 cm³ (D).

Growth Inhibition of Melanoma Cells in Vitro. Growth inhibition of BRO melanoma cells by 9NC in vitro is shown in Fig. 7 for three different concentrations (Fig. 7, B, C, and D). Control cell cultures did not receive treatment (Fig. 7A) or received vehicle alone (PEG); other cultures received 12NC (Fig. 7E). Untreated BRO cell cultures have a doubling time of about 24 hr. A concentration of 9NC as low as 1 ng/ml of culture medium completely inhibited melanoma cell growth (Fig. 7B). At 1 ng/ml 9NC, the actual concentration of PEG in culture is 0.02% (v/v). PEG alone added to BRO cell cultures at a concentration of 0.02% did not inhibit cell proliferation (Fig. 7E). Also, 12NC did not inhibit cell proliferation, even at a concentration of 25 ng/ml (Fig. 7E). CAM and 9AC inhibited BRO cell proliferation at concentrations of 1 ng/ml (growth curves not shown). Treatment of BRO cells with 3 ng/ml and 10 ng/ml 9NC resulted in increased antiproliferative effect (Fig. 7, C and D). Prolonged treatment with 9NC resulted in detachment of BRO cells, with the higher concentrations inducing faster detachment. Therefore, 9NC concentrations above 1 ng/ml were not used in further studies of BRO cells in vitro. We also investigated the minimum period of drug treatment required for a complete inhibition of cell growth. Cultures of BRO cells were treated with 1 ng/ml 9AC and 9NC for various periods of time, and then drug-containing medium was replaced with drug-free medium. The cells were incubated in drug-free medium for 6 days, harvested, and counted. The results demonstrated that a period of 20–24 hr of drug treatment was required for complete growth inhibition of BRO cells. Finally, normal human melanocytes were treated with 1 ng/ml 9AC or 9NC for 6 days. The doubling time for these melanocytes is about 72 hr. No significant difference was observed in the growth rate of treated and untreated melanocytes in a 6-day period.

Microscopy of Cells Treated with CAM Derivatives. Fig. 8 shows the effect of 9NC on BRO melanoma cells in vitro as a function of period of treatment. Cells were photographed prior to addition of 9NC or PEG (Fig. 8a) and after treatment with 1 ng/ml 9NC (Fig. 8 b-e) and PEG (Fig. 8f). Treatment with 9NC for 24 hr resulted in enlargement of the cells and their nuclei (Fig. 8b). As 9NC treatment continued for 3 days (Fig. 8c) and 4 days (Fig. 8d), the enlargement of the attached cells and their nuclei continued. In addition, after 96 hr of treatment with 9NC, about 50% of the enlarged cells possessed two or three nuclei. After 6 days of treatment, >98% of the remaining cells exhibited increased size, with about 30% of the total attached cell population containing three or more nuclei per cell (Fig. 8e). These oversize cells were calculated to be 25–40-fold larger than the untreated BRO cells shown in Fig. 8a. No further enlargement of cells was observed in cultures treated with 9NC for 8 days, but vacuolization appeared in the cytoplasm of many cells after 9 days of treatment. Similar responses were exhibited by BRO cells treated with 9AC at 1 ng/ml (data not shown). In contrast, no morphological changes were observed in the melanoma cells treated with 20 ng/ml 12NC for 10 days (data not shown) and PEG alone for 6 days (Fig. 8f).

Fig. 5. Body weight during tumor regression and in control animals. Measurements of body weight (in g) accompanied measurements of tumor sizes shown in Fig. 4. Measurements also included mice that were not inoculated with the tumor and were treated with the vehicle alone (A) or received drug treatment (B), mice that were inoculated with BRO cells but received no drug treatment (C), and mice that were inoculated with BRO cells and received drug treatment when the tumor size was palpable (D), 0.5 cm³ (E), or 1 cm³ (F).
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Fig. 6. Microscopy of histological sections of BRO tumor. Sections were prepared from tumors surgically removed from mice before treatment with 9NC (a), after 3 days of 9NC treatment (b), or after 14 days of 9NC treatment (c).

In parallel experiments, melanocytes were treated with 1 ng/ml CAM, 9NC, or PEG alone for 5 days, and the cells were observed for morphological changes. Photomicrographs of this experiment are shown in Fig. 9. Melanocytes do not appear to respond to CAM (Fig. 9b) or 9NC (Fig. 9c) with morphological changes, and the majority of the treated cells demonstrate typical slim bodies with two or three dendrites. Further, no changes were observed in melanocytes treated with PEG alone (Fig. 9d). In general, melanocytes treated with CAM, 9NC, or PEG alone were morphologically identical to the untreated melanocytes (Fig. 9a).

DISCUSSION

In this report, we have shown that CAM and its derivatives 9AC and 9NC inhibit growth of human malignant tumors xenografted in immunodeficient mice. In general, chemotherapy for patients with malignant melanoma has been very disappointing, with only a few single chemotherapeutic agents being found to have response rates of 10% or greater in the treatment of metastatic melanoma (23). Continuing research to find new drugs with activity against melanoma has met with little success (23).

In the studies reported here, mice inoculated with human melanoma xenografts were all dead within about 2 months after inoculation, apparently because of development of oversize invasive tumors. However, inoculated mice that were treated with CAM or CAM derivatives were tumor-free. Further, CAM and its derivatives halted progression of already developed melanoma tumors and resulted in tumor regression. The drugs were most effective on small tumors. In previous clinical trials, patients with malignant melanomas were treated i.v. with the water-soluble sodium CAM, but the low antitumor activity and

Fig. 7. Growth of BRO melanoma cells treated with CAM derivatives in vitro. Culture flasks were seeded with $1.5 \times 10^5$ BRO cells. After overnight incubation at 37°C, the medium was replaced with fresh medium, and additives were added. Cells were counted every 2 days after the addition of drugs. Each point represents the average measurement of three flasks. The cultures received no treatment (A), 1 ng/ml 9NC (B), 3 ng/ml 9NC (C), 10 ng/ml 9NC (D), 25 ng/ml 12NC (E, A), or PEG alone (E, B).
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Fig. 8. Microscopy of BRO cells treated with 9NC in vitro. Cell cultures were seeded as described in Fig. 7 and then received 9NC to a final concentration of 1 ng/ml. Stained cultures were microphotographed prior to addition of 9NC (a) and following treatment with 9NC for 1 day (b), 3 days (c), 4 days (d), and 6 days (e). Control cells received PEG alone for 6 days (f). All microphotographs were taken at the same magnification. Bar, 50 μm.

occurrence of severe toxicity (12) led to discontinuation of sodium CAM. This same ineffectiveness of sodium CAM was observed in our studies with nude mice bearing human tumors when the drug was administered i.v. or i.m. (24). In contrast, p.o. or i.m., but not i.v., administration of the non-water-solu-ble CAM prevented growth of human solid tumors in mice, with the effectiveness depending on the mode of administration (24). In general, our studies have indicated that the type of modification of the CAM molecule, the solvent used, and the mode of administration are important factors in effectively...
preserving and applying the antitumor activities of CAM and its derivatives (24).

The inhibitory activity of CAM, 9NC, and 9AC on growth of BRO tumors in nude mice contrasts remarkably with the action of other anticancer drugs. Specifically, we have shown previously that BRO tumors in nude mice do not respond to several widely used anticancer drugs, including Adriamycin, 5-fluorouracil, methotrexate, Alkeran, vincristine, vinblastine, methylchlohexylychloethylnitrosourea, and bischloroethylnitrosourea (25). Although CAM, 9NC, and 9AC consistently exhibit inhibitory activities for tumor growth and cell proliferation, we have used 9NC more extensively than the other products. We have considered 9NC as the derivative of choice for all practical purposes, for the following reasons. (a) The antitumor activity of 9NC in vivo appears to be higher than that of CAM and about equal to that of 9AC. Comparison of drug activities was made on the basis of equal drug doses administered in mice bearing tumors of similar size. (b) 9NC as a powder or in suspension appears to be more stable than 9AC when subjected to environmental agents, including light, oxygen, and water. In the presence of these agents, 9AC generates more rapidly degradation products that are toxic to mice and cultured cells. (c) Semisynthesis of 9NC is simpler than that of 9AC, and thus 9NC can be prepared with a higher yield at a lower cost. Studies on stability of CAM and its derivatives and correlation with antitumor activities and toxicities will be reported elsewhere.4 The mechanism of action of CAM and CAM derivatives is being investigated. It has been suggested that CAM exerts its action at the level of DNA relaxation, by stabilizing the cleavable complexes generated by topoisomerase I and thus interfering with the process of DNA breakage-reunion (8).

The results reported in this study indicate that CAM and its derivatives 9AC and 9NC effectively inhibit growth of melanoma cells at a concentration of 1 ng/ml. This concentration is 500–25,000-fold lower than the CAM concentrations reported to induce DNA strand breaks and cytotoxic effects in mammalian cell lines (3–6). Drug concentrations lower than 1 ng/ml did not completely inhibit cell proliferation but resulted in down-regulation or up-regulation of specific mRNA expression associated with cell growth or cell differentiation.5 In contrast, concentrations of 1–5 ng/ml had no apparent effect on the normal counterparts of the melanoma cells, the melanocytes, in vitro. Melanocytes grow slowly in vitro, and it could be argued that melanoma and other malignant cells proliferate rapidly and, therefore, are more actively involved in the process of DNA breakage-reunion that is the process targeted by CAM. However, recent findings in our laboratory show that CAM and its active derivatives inhibit proliferation of malignant cells regardless of the rate of proliferation.6 It is still unknown whether there is a correlation between the antiproliferative activity of CAM and levels of expression of topoisomerase I. Also of interest is the finding that the CAM derivative 12NC is ineffective in inhibiting malignant cell proliferation in vitro and in vivo. The inactive derivative 12NC and the active derivative 9NC differ only in the position the NO2 group occupies in the CAM molecule. It is likely that a specific molecular conformation is required for CAM and its active derivatives to bind to the DNA cleavable complex. Also, the studies of CAM and CAM derivatives in melanocytes (reported here) and actively dividing normal fibroblasts7 suggest that CAM and its active derivatives exert their action through factors and/or mechanisms not shared by malignant and normal cells. This observation could be of interest when CAM derivatives are considered as chemotherapeutic agents for the treatment of tumors in vivo. It is also of interest to investigate whether pre-malignant cells respond to CAM derivatives like malignant cells. For this, we have extended our studies on human normal cells that have been converted to premalignant cells by insertion of specific oncogenes.

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REFERENCES


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