ABSTRACT

Carbetimer, an intermediate molecular-weight-derivatized copolymer of maleic anhydride and ethylene, has been shown to possess significant antineoplastic activity in the stem cell assay. We have examined the antitumor activity of carbetimer in vivo and in vitro against HMS-Carb/S and M21, both primary human melanoma cell lines sensitive and resistant to carbetimer, respectively. The mechanism of action of carbetimer in HMS-Carb/S has been determined. Mice bearing palpable sensitive tumors were treated with 10% lethal doses of carbetimer (1500 mg/kg i.p.). The tumor nucleotide profile was determined 4 hours later. Uridine and cytidine nucleoside triphosphates were reduced by 36.6 and 58.2%, respectively. In a similar experiment using carbetimer-resistant tumor, there was no change in the tumor pool sizes of uridine and cytidine nucleoside triphosphate pools in carbetimer- or saline-treated animals. Following 24-h exposure of the cells to 1000 µM concentration of carbetimer, the carbetimer-sensitive cells were pulsed with [14C]uridine, cytidine, or thymidine for 30 min. Pyrimidine nucleotides, in particular triphosphates, were reduced significantly as compared to the saline-treated control. Similar treatment of carbetimer-resistant cells resulted in no change in the pool sizes of the nucleotides. [14C]Carbonate flux studies demonstrated that [14C]CO2 conversion into UMP and CMP was increased 200 and 140% of control in the carbetimer-sensitive cells treated with 1000 µM carbetimer; however, a similar treatment of the resistant cells showed no change in the pool sizes of the nucleotide. Examination of pyrimidine salvage enzymes demonstrated that, in the sensitive cells, carbetimer treatment reduced the specific activity of uridine, cytidine, and thymidine kinase by 46, 37, and 60%. In a similar study using resistant cells, the specific activities were reduced 7 and 0%, respectively. In the restitution studies coinoculation of carbetimer-sensitive cells with carbetimer and uridine resulted in essentially the reversal of carbetimer cytotoxicity. Thus, carbetimer inhibits the growth of the sensitive cells by inhibiting the uptake and metabolism of preformed nucleosides both in vivo and in vitro.

INTRODUCTION

The antineoplastic activity of polymeric compounds has been a matter of interest since Regelson et al. (1, 2) documented that certain synthetic polyelectrolytes possess antineoplastic activity against a number of animal tumors. Carbetimer is a low-molecular-weight polymer derived from ethylene and maleic anhydride (3). The compound has demonstrated activity against Lewis lung, Madison 109 lung tumors, and colon 26 tumor in mice (4). Moreover, using the human stem cell assay, Kiser et al. showed that carbetimer is active against a number of human tumors (5). Response was noted in 15 out of 53 tumors and antitumor activity was seen in 4 out of 19 breast cancer specimens and in 4 out of 17 ovarian cancer specimens. In our laboratory, we have developed an in vivo nude mouse model to test the activity of carbetimer. In approximately 50% of established human tumors in nude mice, carbetimer has shown activity. Carbetimer is undergoing phase I clinical trials and as such, the drug is undergoing phase I clinical trials and is considered a potential drug for further clinical trials.

MATERIALS AND METHODS

Chemicals. [2-14C]Uridine (30 mCi/mmol), [2-14C]thymidine (60 mCi/mmol), deoxy-[2-14C]uridine (39 mCi/mmol), deoxy-[2-14C]thymidine (32 mCi/mmol), and sodium [14C]bicarbonate (57.5 mCi/mmol) were obtained from Amersham/Searle Corp. (Arlington Heights, IL). In Vitro Cytotoxicity. HMS-Carb/S, M21, human melanoma cell lines were grown in RPMI 1640 containing 5% (v/v) fetal calf serum, 0.06% (w/v) L-glutamine, penicillin (62 mg/l), and streptomycin (135 mg/l) at 37°C in an atmosphere of 5% CO2/95% air. For the initiation of cell growth experiments, logarithmically growing cells were harvested with Dulbecco’s phosphate-buffered saline containing 0.05% trypsin and 0.02 mM EDTA and were suspended in medium plus serum; 5 x 10^5 cells were plated in 75-cm² plastic tissue culture flasks. After allowing the cells to adhere to the flask for 1 day, the medium was decanted and replaced with fresh medium containing 5% dialyzed fetal calf serum with carbetimer in a range of 100–1000 µM. One, 3, and 5 days after drug treatment, the cells were washed once with Dulbecco’s phosphate-buffered saline, trypsinized, and counted in an FACS Coulter Counter. Results are expressed as percentage of control cell count.

In Vivo Transplantation of Human Melanoma. HMS-Carb/S and M21 cells were grown in vivo. Between 10–100 million cells were injected into the flanks of BALB/c nude mice. Four weeks later, murine tumors achieved sizes between 0.5 and 1.0 cm across, at which time tumor inoculum was prepared. The 100–1000 µM was used in each group and the remaining animals served as controls. Thereafter, mice were examined twice weekly. The perpendicular diameters of the tumors were measured with the aid of a caliper and were converted to weight by the following:

\[ \text{Tumor weight (mg)} = \frac{a \times b^2}{2} \]

where a and b are both in mm (6).

Influence of Carbetimer Treatment on the Pool Sizes of Ribonucleotides in Nude Mice Bearing Human Melanoma. Two human tumors with known sensitivity or resistance to carbetimer were used in this study. HMS-Carb/S represented a carbetimer-sensitive tumor and M21 represented a carbetimer-resistant melanoma. Mice, each bearing a 0.5–1-cm tumor, were used. Groups of 3 mice in each group were given i.p. injections of 0.9% saline solution or carbetimer (1500 mg/kg). Four h later the mice were lightly anesthetized with ether, and the tumor was freeze-clamped, subsequently pulverized with a percussion hammer, and homogenized at 4°C in 5% perchloric acid (1:3, tumor/perchloric acid, w/v) and immediately neutralized. The neutralization procedures were as follows: To 1 ml of homogenate was added 50 µl of 1 M potassium phosphate, pHe 8.5, and 70 µl of 40% KOH to bring the
mixture to pH 7.0–7.2. The neutralized homogenate was immediately frozen on dry ice until it was analyzed for the tumor nucleotides. The separation of nucleotides were achieved by high-pressure liquid chromatography using an Altex Model 420 system (Altex Instruments, Berkeley, CA), equipped with a Partisol-10 SAX column (0.45 x 25) (Whatman, Inc., Clifton, NJ). Separation was accomplished by elution for 10 min at 1 ml/min with 0.005 M KH₂PO₄, pH 2.8, after which the flow rate was increased by 2 ml/min, and a linear gradient of 0.005 M KH₂PO₄, pH 2.8, to 0.5 M KH₂PO₄, pH 4.8, was maintained over 20 min. Absorbance was monitored at 254 nm, and a fraction of eluate was collected every 30 s (7).

Incorporation of [¹⁴C]Uridine to Respective Nucleotide by Human Melanoma Cells. Exponentially growing HM5-Carb/S and M21 cells (1 day after plating 5 x 10⁶ cells) were incubated for 4 or 24 h with either carbetimer, 1 x 10⁻³ M, or saline. At predetermined times cells were washed and incubated for a further 1 h with 20 µCi [¹⁴C]Uridine. Thereafter, the cells were washed twice with phosphate-buffered saline and trypsinized, and the cell pellet was homogenized in 5% (w/v) ice-cold perchloric acid. The procedure for nucleotide separation is mentioned above.

Incorporation of [¹⁴C]Uridine to Respective Nucleotides in Vivo. Mice bearing human melanoma sensitive and resistant to carbetimer (HM5-Carb/S and M21, respectively) were treated with carbetimer, 1500 mg/kg, i.p., or saline control. Four h later, each animal was administered 50 µCi of [¹⁴C]Uridine i.p. and 1 h later animals were sacrificed and the tumors were prepared for loading on high-pressure liquid chromatography as mentioned above.

[¹⁴C]Bicarbonate Incorporation into Cells. Flasks (150 cm²) containing 5 x 10⁶ logarithmically growing cells were rinsed twice with HCO₃⁻-free Eagle's Medium no. 2 containing 2 x 1-glutamine, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 10% dialyzed fetal calf serum. To begin an experiment, 10 ml of the aforementioned medium, with or without 1 x 10⁻³ M carbetimer, was added to the flasks. After a 3-h incubation, 200 µCi of [¹⁴C]NaHCO₃ was added to each flask, and the cells were incubated for an additional 3 h. Following this incubation, the medium was decanted (the unincorporation radiolabel was trapped in alkali for subsequent isolation of the media), and the cells were trypsinized, washed, pelleted, and lysed by the addition of 1 ml ice-cold 3% HClO₄. Acid-soluble supernatants were chromatographed on an anion-exchange column; 2.5-min fractions were collected and counted (8).

Pyrimidine Salvage Enzymology. Effect of carbetimer on the in vitro pyrimidine nucleoside kinase activity as carried out by incubating at 37°C for 15 min in a reaction mixture consisting of 5 µl of [³H]labeled precursor (0.25 µCi; 5 nmol), 5 µl of 0.01 M ATP-MgCl₂ in 0.05 M Tris, pH 8.4, or buffer alone, and 5 µl of tumor supernatant. The reaction was terminated by adding 10 µl of 2 M HCl followed by centrifugation at 12,000 × g for 3 min. Samples (5 µl) were subjected to ascending paper chromatography on Whatman no. 3M paper for 18 h using isopropyl alcohol/EDTA (saturated solution)/toluene/14 M NH₄OH (320/44/40/4) as solvent (8). The phosphorylated products are retained at the origin.

Protection Studies. HM5-Carb/S and M21 cell lines were tested with increasing concentrations of carbetimer (200–1000 µM) with or without the addition of uridine (500 µM). After 48 h, the cells were harvested and counted as before.

RESULTS

Sensitivity or Resistance to Carbetimer in Vivo. One human melanoma cell line, HM5-Carb/S, is sensitive to carbetimer, whereas M21, another human melanoma, is resistant to the agent. The treatment of nude mice bearing the s.c. implanted tumor with carbetimer led to a decrease in the tumor growth in mice bearing the carbetimer-sensitive tumor (HM5-Carb/S) while no such tumor inhibition was seen in mice bearing the M21 tumor resistant to carbetimer (Table 1). By day 30–35 all of the control and mice bearing carbetimer-resistant tumors had died, whereas 100% of mice bearing carbetimer-sensitive tumors were alive with minimal tumors. By day 60, mice bearing carbetimer-sensitive tumors, demonstrated large tumors.

Effect of Carbetimer on the HM5-Carb/S Growth and M21 Cell Lines in Vitro. Logarithmically growing cells were exposed to several concentrations of carbetimer and the cell number was determined after 1, 3, and 5 days. HM5-Carb/S cells demonstrated sensitivity to carbetimer—at 1000 µM, greater than 50% inhibition was seen. With the M21 melanoma cell line, carbetimer, at its highest test concentration, had no effect on the inhibition of cell growth (Fig. 1).

Influence of Carbetimer Treatment on the Pool Sizes of Ribonucleotide Triphosphates in Nude Mice Bearing Carbetimer-sensitive and -resistant Melanoma Cell Lines. Four hours following treatment with carbetimer of tumor-bearing mice, tumor nucleotide pools were determined (Table 2). Carbetimer treatment did not alter the pool sizes of purine nucleotides; however, there was a substantial and significant decrease in the pool sizes of UTP and CTP in the carbetimer-sensitive melanoma cell line. The carbetimer-resistant melanoma cell line demonstrated no significant change in the pool sizes of either purine or pyrimidine nucleotide triphosphates.
Incorporation of [1^4C]Uridine to Respective Nucleotides by Human Melanoma Cells in Vitro and in Vivo. Since carbetimer had a profound effect on the uridine and cytidine nucleotide, it was interesting to examine the influence of [1^4C]uridine uptake and nucleotide formation in the carbetimer-sensitive and resistant lines. The [1^4C]UMP pool in the sensitive cells treated with carbetimer demonstrated a significant reduction compared with the saline controls. [1^4C]UMP and UDP did not show significant pool size changes. In the carbetimer-resistant cell line, the carbetimer total did not alter the pool sizes of the labeled nucleotides (Table 3).

In concert with in vivo studies, our in vitro experiment showed that nude mice bearing carbetimer-sensitive and resistant melanoma demonstrated similar [1^4C]uridine nucleotide pool changes. (Results are not shown.)

Effect of Carbetimer on the Pyrimidine Salvage Enzymology. Since our in vivo and in vitro base studies demonstrated that carbetimer suppresses the pyrimidine nucleotide formation, it was pertinent to know the effect of carbetimer on the pyrimidine nucleoside enzymes. As is shown, carbetimer reduced the activity of pyrimidine nucleoside kinases in the sensitive cell; however, no such change was seen in the carbetimer-resistant line (Table 4).

Bicarbonate Flux Studies. Carbetimer treatment of HM5-Carb/S cells and subsequent treatment with [1^4C]bicarbonate resulted in the increased formation of nucleotides via the pyrimidine de novo pathway. Therefore, UMP and CMP were increased 200 and 140%, respectively, as compared with the controls (Fig. 2). Similar treatment of the resistant cells showed no change in the pool sizes of the nucleotides compared with the controls.

Protection Studies. Protection of cytotoxicity following coadministration or uridine with carbetimer is shown in Fig. 3, in
which addition of increasing doses of carbetimer in HM5-Carb/S cell lines in culture inhibited the cellular growth; however, addition of uridine (500 μM) reversed the antitumor activity of carbetimer. In the resistant cell line M21, carbetimer had no effect on the cellular inhibition. (Results not shown.)

DISCUSSION

This is the first study of the mechanism of action of a new intermediate molecular weight polymer, carbetimer. For the purpose of this study we have identified two melanoma cell lines, one sensitive to carbetimer, HM5-Carb/S, and one resistant to carbetimer, M21. The cells were successfully transplanted into the flanks of nude mice. Carbetimer treatment inhibited the growth of the sensitive tumors and, by day 30–35, 100% of animals were alive as compared to the saline control, in which all mice had died. In contrast, in carbetimer-resistant tumors, 50% of the treated and saline control only were dead by day 30–35. In vitro experiments similarly demonstrated concentration-dependent activity of carbetimer in the sensitive cells whereas the resistant cells were totally resistant to carbetimer. In vivo carbetimer treatment of mice bearing the sensitive tumors demonstrated that the pyrimidine triphosphate nucleotides were substantially reduced; however, this was not seen in the resistant tumors. In the subsequent in vivo and in vitro studies we have demonstrated that uptake and incorporation of [14C]uridine into nucleotides is reduced by the coadministration of carbetimer. [14C]Thymidine and cytidine were similarly inhibited by carbetimer. Therefore, it is conceivable that carbetimer may be inhibiting uptake and/or phosphorylation of the pyrimidine nucleosides. The examination of the pyrimidine salvage enzymology demonstrated inhibition of pyrimidine nucleoside kinase in the carbetimer-sensitive cell line, and no inhibition in the resistant line. Finally, in our in vitro studies using carbetimer-sensitive cells, we have shown that the addition of uridine to the culture system essentially reversed the activity of carbetimer. We believe that carbetimer is a membrane-active drug and may indeed interfere with other substrates besides nucleosides. However, we have shown in this manuscript that the inhibition of nucleoside by carbetimer is cytotoxic and, furthermore, reversal of this phenomenon will lead to reduced cytotoxicity by carbetimer.

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Mechanism of Action of a New Antitumor Agent, Carbetimer

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