Conversion of highly malignant colon cancer from an aggressive to a controlled disease by oral administration of a metalloproteinase inhibitor

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In this study, we describe the activity of CT1746, an orally-active synthetic MMP inhibitor that has a greater specificity for gelatinase A, gelatinase B and stromelysin than for interstitial collagenase and matrilysin, in a nude mouse model that better mimics the clinical development of human colon cancer. The model is constructed by surgical orthotopic implantation (SOI) of histologically-intact tissue of the metastatic human colon tumor cell line Co-3. Animals were gavaged with CT1746 twice a day at 100 mg/kg for 5 days after the SOI of Co-3 for 43 days. In this model CT1746 significantly prolonged the median survival time of the tumor-bearing animals from 51 to 78 days. Significant efficacy of CT1746 was observed on primary tumor growth (32% reduction in mean tumor area at day 36), total spread and metastasis (6/20 treated animals had no detectable spread and metastasis at autopsy compared to 100% incidence of secondaries in control groups). Efficacy of CT1746 could also be seen on reducing tumor spread and metastasis to individual organ sites such as the abdominal wall, cecum and lymph nodes compared to vehicle and untreated controls. We conclude that chronic administration of a peptidomimetic MMP inhibitor via the oral route is feasible and results in inhibition of solid tumor growth, spread and metastasis with increase in survival in this model of human cancer, thus converting aggressive cancer to a more controlled indolent disease.

Keywords: CT1746, matrix metalloproteinase inhibitors, MMPs, tumor growth

Introduction

Malignancy is most often a very aggressive disease with a rapid course leading to the demise of the patient. However the present therapeutic modalities can not successfully control most solid tumors with rapid local invasive growth and metastasis, contributing to the poor 5-year survival rate. An alternative measure to this enormous problem is to convert human malignancy from a highly aggressive to a more controlled indolent disease that allows the patient to survive longer with minimum symptoms. Targets for this therapeutic approach include the matrix metalloproteinases (MMPs), which are a family of zinc-dependent endopeptidases frequently found in and around the more invasive and metastatic human tumors [1]. They possess proteo-
lytic activity for components of the extracellular matrix such as collagen and proteoglycans and are thought to promote the growth and spreading of tumor tissue, possibly through degradation of basement membranes, the modulation of sites of cell adhesion, and by facilitating tumor angiogenesis [1, 2]. Genetic manipulation of MMP expression, or studies involving one or other of the recombinant tissue inhibitors of metalloproteinases (TIMP1 and TIMP2), have demonstrated the involvement of these enzymes in the process of tumor growth and metastasis [3–5]. More recently, efficacy has been demonstrated with synthetic MMP inhibitors in animal models of tumor growth and spread [6, 7]. Further more, it has been recognized since early this century that solid tumors possess an abnormal blood supply [8] and it is of considerable interest that synthetic MMP inhibitors are also inhibitors of angiogenesis [9, 10].

Targeting tumor vasculature is an example of an approach to cell control as opposed to the traditional approach of cell kill [11]; as such, the pharmacology of this type of therapeutic agent is completely different from that of conventional agents. For example, chronic dosage regimes maintained for long periods appear necessary and in consequence orally active compounds are mandatory.

One of our laboratories (AntiCancer Inc.) has developed a nude mouse model that better mimics the clinical development of human colon cancer by surgical orthotopic implantation (SOI) of histologically intact human tumor tissue [12, 13]. Orthotopic transplantation of histologically intact tissue enables the model to reflect the clinical behavior of human cancer including primary tumor growth, invasion and metastasis [14, 15]. Using this model, the present study examined the anti-tumor and anti-metastatic efficacy on chronic administration of an MMP inhibitor, CT1746, which is known from screening studies [16] to be absorbed after oral administration. The results described here demonstrate that malignant colon cancer, treated chronically by oral administration with CT1746 in the model, could be converted from a highly aggressive to a more controlled indolent disease with significant increase in survival.

Materials and methods

Animals

A total of 55 male 3- to 4-week-old, nu/nu CD-1 outbred mice (Charles River Laboratory, Wilmington, MA), were used in the study. They were kept under specific pathogen-free conditions. The animal diets were obtained from Harlan Teklad (Madison, WI) and 0.15% (v/v) HCl was added to the drinking water.

Colon carcinoma xenograft

A metastatic human colon cancer cell line, Co-3, was used in this study. This well-established cell line was kindly provided by Dr Tetsuro Kubota (Keio University, Tokyo, Japan). It is a well-differentiated adenocarcinoma of the colon and was obtained from a metastatic lesion in the lung of a 39-year-old female patient in 1975. Co-3 has a rapid yet stable growth rate and has shown no changes in histology from the original tumor, even after repeated transfers [17]. The cell line has been maintained subcutaneously in nude mice in Keio University as well as our animal facility. The specimen for orthotopic implantation was derived from tumor stock growing subcutaneously in nude mice.

Gelatinolytic activity was detected in cytosols prepared by disrupting pieces of normal caecum, or orthotopically grown tumor tissue, with a Mikro-Dismembrator U (B. Braun Biotech, Melsungen, Germany). This machine disrupts frozen tissue by shaking it rapidly in a flask with a tungsten ball. Each piece of tissue was weighed and similar amounts were disrupted by the flask being shaken at 1600 rpm for 2 min. The tissue was allowed to thaw before the addition of 0.1 M Tris base, 0.1 M NaCl, 0.05% Tween 20, pH 7.4, to give a final ratio of 1:5 tissue to buffer (w/v). The solution was then centrifuged in an Eppendorf microfuge for 2 min, the supernatant was removed and the protein content determined using the Coomassie Protein Reagent (Pierce and Warriner). The gelatinolytic content was evaluated by analysing equal amounts of total protein by gelatin zymography [18]. The sensitivity of any gelatinase activity to inhibition by CT1746 was determined by including the inhibitor at a concentration of 1–100 nM during the incubation step.

Construction of orthotopic model

The stock tumor tissue was derived from subcutaneously-growing tumors when they were in the log phase. Fresh tumor tissue was harvested from the periphery of the tumor masses. The tumor tissues were then minced in RPMI-1640 culture medium with antibiotics into 1 x 1 x 1 mm³ fragments. The resulting fragments were then randomized. Orthotopic implantation of Co-3 human colon cancer tissue was carried out using micro-surgical procedures we have developed previously [12–15].
Briefly, mice were anesthetized by isoflurane inhalation and immobilized in a supine position. With the aid of a dissecting microscope (×7) a small midline incision was made in the abdomen and the coloceleal portion of the colon was exposed. A small piece of serosa was then removed from the site where the tumor fragments were to be implanted and 10 fragments were implanted on the top of the intestine using 8-0 nylon sutures. The intestine was returned to the abdominal cavity and the abdomen was closed with 6-0 surgical sutures.

The tumor fragments were randomized such that quantitatively and qualitatively equal amounts of tumor were implanted in each mouse so as to limit the mouse to mouse variability in subsequent tumor growth within a statistically acceptable range.

The mortality rate of surgical procedure is approximately 5%, which was mainly due to anesthesia overdose. The other cause of postsurgical death is abdominal wall rupture. Routinely several extra mice are transplanted to compensate for possible postsurgical loss.

**Synthetic inhibitor of MMP**

CT1746 (N1-[2-(S)-(3,3-dimethylbutanamidyl)-N4-hydroxy-2-(R)-[3-(4-chlorophenyl)-propyl]-succinamide) is a recently described orally active peptidomimetic MMP inhibitor which incorporates a zinc chelating hydroxamic group within a structure that resembles a peptide substrate [6, 16, 19-21]. Selectivity towards gelatinase was obtained by including an arylpropyl group at the $P_1'$ position, which mimics the aromatic amino acid usually found at the $S_2'$ position in the MMP propeptide that is cleaved during autoactivation [22]. Cleavage at this position by collagenase does not readily occur [23] and as a result CT1746 exhibits a significantly greater selectivity for gelatinase than for collagenase.

The compound is insoluble in aqueous solution and for kinetic analysis was dissolved in methanol before dilution in assay buffer. For *in vivo* experiments it was formulated in propylene glycol as described below. The $K_i$ values using a quenched fluorescent peptide substrate [16, 18] against human gelatinase-A, gelatinase-B, stromelysin 1, collagenase, and matrilysin are 0.04, 0.17, 10.9, 122 and 136 nM, respectively. Against other classes of metalloproteinases such as neprilysin (EC 24.11), meprin, peptidyl-dipeptidase A (ACE), and aminopeptidase N, CT1746 exhibits negligible activity with IC$_{50}$ values against peptide substrates that are greater than 40 µM (unpublished observations). We confirmed *in vitro* that the compound could inhibit the gelatin degrading activity of gelatinase A; and when administered orally to mice at 100 mg/kg in a volume of 0.2 ml, a single dose could inhibit gelatin degradation *in vivo* in a time-dependent fashion, with 50% inhibition being recorded after 12 h [16].

Acetonitrile precipitation of plasma extracted at various time points from mice that had been dosed as described above, followed by HPLC analysis, revealed a peak plasma concentration of 25 µM at 20–40 min, after which the concentration declined to approximately 2.5 µM. This concentration was maintained for at least 8 h, and we conclude that the propylene glycol facilitates a slow absorption rate, resulting in the maintenance of circulating levels of inhibitor for long periods of time. This conclusion is supported by the biodistribution of $^{14}$C-labeled CT1746 which was found to exhibit a long residency time in the stomach and intestines, with relatively lower levels in other tissues. Confirmation that the CT1746 detected by HPLC was active was obtained by methanol precipitation of plasma followed by its titration against gelatinase A in the quenched fluorescent peptide assay.

No evidence of any CT1746-associated cytotoxicity in assays of clonogenicity, thymidine incorporation, or measurement of cell viability by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) staining was observed. Colorectal cell lines of both murine (COLO26) [24] and human (SW1222) [25] origin were tested, and inhibitor concentrations ranging from 0.08 to 10 µM over 3–7 days were employed. In the MTT assay, exposure to 25 µM CT1746 for 4 days had no measurable effect on HT1080 [3] cell viability.

Less favorable pharmacokinetics were achieved at a lower dose of 10 mg/kg, with the compound being undetectable in the plasma after 30 min by these methods. A dose of 100 mg/kg was therefore selected for the efficacy studies.

**Experimental design**

Fifty-five mice were randomly assigned to the following three groups on day 2 after surgical orthotopic implantation (SOI) of the human colon tumor: untreated control (15 mice); CT1746 (100 mg/kg bd; 20 mice); vehicle (propylene glycol) control (0.2 ml bd; 20 mice).

The vehicle and drugs were administered orally by gavage using an animal feeding needle (22 guage, Pro Vet Inc., City of Industry, CA). CT1746 was administered at a dosage of 100 mg/kg in propylene glycol twice a day with a 12-h interval. Each mouse received a volume of 0.2 ml of drug suspension in a single dose. Administration of compound started 5 days after SOI.
All mice were kept under close observation for symptoms of tumor growth and spread. Body weight, tumor size and survival information were collected during the course of the experiment. With regard to the compound and vehicle groups, gavage and data collection were conducted in a blinded fashion.

All mice, when found dead, were immediately immersed in 10% neutral buffered formalin for subsequent macroscopic and microscopic examination. Tissue samples derived from the primary tumor, the spread found on the colon, the cecum, the ileum and the abdominal wall, as well as the tissues of the lung, the liver and the regional lymph nodes were processed for histopathology study from all the groups. Standard H&E staining was used for microscopic examination of all the tissue sections.

Measurement of primary tumor size and animal body weight
Primary tumor size during the course of the experiment was measured with calipers by abdominal wall palpation and the tumor area was calculated by multiplying the two largest diameters. Body weight of the animals was measured by an electrical balance.

Evaluation of tumor spread and metastasis
The term ‘spread’ here stands for spontaneous implantation and direct invasion of the peritoneal surface of the adjacent organs such as the abdominal wall, the cecum and the ileum in the abdominal cavity (Figure 3). Any visible tumor deposit in the abdominal cavity other than the primary tumor was considered as spread, which was determined macroscopically under a dissecting microscope (×7) and confirmed microscopically.

The liver, lung and para-aortic and mesenteric lymph nodes were routinely serially sectioned and stained for microscopic examination of metastasis. In this study, when lymph node metastasis was considered together with spread upon evaluation, they were defined as the ‘secondary tumor deposits’.

Statistical analysis
Differences in median survival, and in mean size of the primary tumors at defined time points, between the three groups were assessed for significance using the Wilcoxon Rank-Sum test and Student’s t-test, respectively. Incidence of survivors at defined time points and incidence of secondary tumor deposits per group as well as the incidence of secondary tumor deposits per organ site in different groups on autopsy were assessed using the Fisher Exact test. The comparison of the average number of sites of secondary tumor deposits (spread and lymph node metastasis) per mouse between groups was assessed using the Mann-Whitney U-test. As used here all tests of significance were two-tailed.

Results
In situ MMP expression by the Co-3 tumor
The gelatinolytic activity of orthotopically grown Co-3 tumor tissue and normal cecum from non-tumor-bearing mice was compared. Frozen explants of each type of tissue were used to prepare cytosols which were analysed by gelatin zymography. Normal cecum generated two major bands of approximately 45 and 55 kDa, whereas the tumor-bearing tissue had additional higher molecular weight bands including a predominant band corresponding in size to murine gelatinase-B. On incubation of the zymograms in the presence of 1, 10, or 100 nM CT1746, it was found that only the additional gelatinolytic bands associated with the tumor samples were inhibited, partially at 1 nM, and completely at 10 and 100 nM, thereby demonstrating selective inhibition of the tumor-associated gelatinase.

Effect of CT1746 on survival
Tumor was palpable in mice of all three groups 13 days after implantation (8 days after initiation of treatment). At this point, animals were observed closely and when clearly symptomatic (showing signs of cachexia and declining or restricted physical activity) due to effects of progressive tumor, or if having succumbed, were assigned a survival time.

By day 41 (36 days of treatment) it was apparent that CT1746 was exerting an effect. Thus, by this time, the incidence of survivors (Figure 1) in the compound-treated group was significantly greater than in control groups (CT1746 vs untreated, \( P = 0.04 \); CT1746 vs vehicle, \( P = 0.03 \) by the Fisher Exact test). Consequently, response to treatment was consolidated for a further 7 days before gavage was stopped.

The last animal was sacrificed on day 136 and the survival curve, accounting for all subjects in the study, is shown in Figure 1. The median survival time in the three groups is as follows: untreated, 51 days; CT1746, 78 days; vehicle, 43 days. There was no significant difference between control groups; however, there were highly statistically significant differences in median survival between the compound-treated group and control group (CT1746 vs untreated, \( P = 0.0002 \); CT1746 vs vehicle, \( P = 0.0001 \).
The early demise of two to three mice in the vehicle control group (as shown in Figure 1) might have been due to the gavage procedure, which the untreated control group was not subjected to. It should be noted that despite the gavage procedure the CT1746-treated group had a much greater survival, further indicating the strong efficacy of CT1746.

Effect of CT1746 on primary tumor growth, spread and metastasis

The rate of primary tumor growth was serially assessed over 56 days by palpation and measurement of abdominal tumor. Figure 2 shows growth delay curves for the CT1746-treated group versus untreated and vehicle-treated groups: for clarity, the comparisons are shown separately. In both cases it can be seen that CT1746 treatment produces a marked growth delay relative to both control groups. At each time point the number of tumors used to calculate each mean tumor size is quoted and it can be seen in the case of the comparison of CT1746-treated with untreated animals that the number of subjects decreases rapidly from day 36 onwards, making statistical comparisons less secure at later time points. Nevertheless, at day 36 CT1746 treatment reduced tumor size by 32% relative to the untreated group ($P = 0.0001$) and by 39% relative to the vehicle-treated group ($P = 0.002$). There was no significant difference between the control groups.

Table 1 shows that in both control groups secondary tumor deposits (including spread to the abdominal wall, cecum and the ileum and metastasis to the mesenteric lymph node) were seen in all evaluable animals whereas in six animals treated with CT1746 lesions they were restricted to the site of implantation. The difference in the total incidence of secondary lesions was statistically significant when the CT1746-treated group was compared with both control groups (Table 1). Quantitative evaluation of the average number of sites of tumor spread and metastasis per mouse also showed advanced tumor development with statistical significance in the control groups compared with the treated group ($P = 0.0021$, untreated control vs CT1746; $P = 0.0031$, vehicle control vs CT1746; see
Figure 2. Growth delay of mice orthotopically implanted with Co-3 tumor after treatment with CT1746. Same experiment as Figure 1. Growth of primary tumor was assessed serially by caliper measurement after abdominal palpation: tumor size was computed from the product of two diameters. Data expressed as mean ± 1 SD. See text for statistical analysis. For clarity, comparisons of primary tumor growth after CT1746 treatment with the control groups are shown separately.
Table 1. Effect of CT1746 on tumor spread and metastasis

<table>
<thead>
<tr>
<th>Treatment (no. of mice)</th>
<th>No. of mice available for autopsy&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of mice without secondary deposit&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Significance relative to untreated&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control (15)</td>
<td>14</td>
<td>0/14</td>
<td></td>
</tr>
<tr>
<td>Vehicle&lt;sup&gt;d&lt;/sup&gt; bd&lt;sup&gt;e&lt;/sup&gt; control (20)</td>
<td>19</td>
<td>0/19</td>
<td>NS</td>
</tr>
<tr>
<td>CT1746 100 mg/kg bd (20)</td>
<td>20</td>
<td>6/20</td>
<td>&lt;sup&gt;P = 0.028&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> One animal in the untreated control and one in the vehicle group were unavailable due to tissue changes postmortem.

<sup>b</sup> See Table 2 for details concerning sites of tumor spread and metastasis.

<sup>c</sup> Differences in the incidence of secondary tumor deposits relative to the untreated control assessed by two-tailed Fisher's exact test. Significance of difference between compound and vehicle control, \( P = 0.012 \).

<sup>d</sup> Vehicle was neat propylene glycol (0.2 ml/dose).

<sup>e</sup> bd = twice daily (at approx. 12 h interval).

Table 2. Effect of CT1746 on the incidence of tumor spread and metastasis at different organ sites

<table>
<thead>
<tr>
<th>Group</th>
<th>Abdominal&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cecum&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ileum&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Mesenteric&lt;sup&gt;d&lt;/sup&gt; lymph nodes</th>
<th>Median with range&lt;sup&gt;e&lt;/sup&gt; for no. of sites of spread and metastasis per mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT1746, 100 mg/kg</td>
<td>12/20 (60.0%)</td>
<td>8/20 (40.0%)</td>
<td>1/20 (5.0%)</td>
<td>0/20 (0.0%)</td>
<td>1(0-2)</td>
</tr>
</tbody>
</table>

<sup>a</sup> P = 0.0504, CT1746 vs untreated control; P = 0.5006, CT1746 vs vehicle control; all by Fisher's exact test.

<sup>b</sup> P = 0.0382, CT1746 vs untreated control; P = 0.0004, CT1746 vs vehicle control; all by Fisher's exact test.

<sup>c</sup> P = 0.99, CT1746 vs untreated control; P = 0.605, CT1746 vs vehicle control; all by Fisher's exact test.

<sup>d</sup> P = 0.0216, CT1746 vs untreated control; P = 0.047, CT1746 vs vehicle control; all by Fisher's exact test.

<sup>e</sup> P = 0.0021, CT1746 vs untreated control; P = 0.0031, CT1746 vs vehicle control; all by Mann-Whitney U-test. See Table 3 for the number of mice under different categories regarding the number of sites of secondary tumor deposit in the three groups.

Table 3. Effect of CT1746 on the number of secondary tumor deposits per mouse

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of sites of tumor spread and metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Untreated control</td>
<td>0</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>0</td>
</tr>
<tr>
<td>CT1746 (100 mg/kg)</td>
<td>6</td>
</tr>
</tbody>
</table>

Tables 2 and 3 for details). Separate analysis of the incidence of secondary tumor deposits on different organs (the abdominal wall, the cecum, the mesenteric lymph nodes) in the different groups also showed statistically-significant differences between the CT1746-treated vs both untreated control groups (see Table 2 for details).

Histopathology studies of the primary tumor in both the CT1746-treated group and the control groups did not show different cytotoxicity (Figure 3). Also no differences were observed in histologic morphology.
Effect of CT1746 on animal body weight
The mean body weight (Figure 4) in the untreated control group increased until day 21, from 17.7 to 21.0 g, while both the CT1746-treated and vehicle control groups showed a slight drop in body weight (from 19.2 to 18.4 g and from 20.0 to 18.1 g, respectively), which may have been due to the gavage procedure. By day 29, mean body weights in all three groups had stabilized at 18-19 g and remained at this level for a further 10 days, by which time treatment effects were impossible to separate from tumor cachexia.

Discussion
Models of surgical orthotopic implantation (SOI) of histologically-intact human tumor tissue in nude mice provide a unique opportunity for assessing activity of new anticancer agents against a variety of tumor types. This model, compared with orthotopic inoculation of cell suspensions and subcutaneous models, can reveal more clinical characteristics of malignant tumors, such as invasive local growth, spread and distant metastasis [12, 13]. Therefore the model can provide a clinical profile that helps in the evaluation of new therapeutic modalities.

The immunodeficient nude mouse has been used in the field of cancer research since the 1960s. Although they have severely reduced numbers of mature functional T lymphocytes, which are critical in tumor immunity, it has been shown that T-cell-like activity can be induced [26, 27], especially when the nude mouse encounters a microbial infection. Also it is believed that the older the mice are, the stronger the residual T-cell function is. Moreover, the activities of other cell types in the nude mouse defense system, such as NK cells and macrophages, are reported to be at normal or even higher levels [28, 29]. These cells are suggested to play an important role in inhibiting tumor xenograft growth in nude mice [30, 31]. Based on these properties, mice of the same strain, same sex and the same age were used for all the groups in this study to eliminate any possible variables in this aspect. Also, mice of young age (3-4 weeks old in this study) were chosen to facilitate tumor growth and to avoid the possible variability of residual T-cell function in older mice.

The salient points emerging from this evaluation of CT1746 are that a non-cytotoxic peptidomimetic compound, administered orally in a chronic fashion, exerts a degree of control on tumor growth, spread and metastasis. That CT1746 is not generally cytotoxic is suggested by the lack of compound-related toxicity as assessed by body weight over the period of administration. In addition, we have found that it has no effect on cell viability in vitro at a concentration as high as 25 μM (see Materials and Methods).

The results herein are noteworthy because, so far as we are aware, this is the first published demonstration of anti-tumor activity using an orally active, synthetic MMP inhibitor. This is important for two reasons: first, although it is frequently possible to synthesize potent peptidomimetic
inhibitors [19–21, 32], they are generally found to possess little or no oral activity. Building this into the molecule is unpredictable and may militate against potency [33]. Second, pharmaceuticals designed to exert cell control rather than cell kill will require chronic administration and this is only feasible with compounds that are absorbed from the gastrointestinal tract.

The tumor response recorded here is manifested as an increase in survival (Figure 1), which is presumably a reflection of reduced primary tumor growth (Figure 2) and lower incidence of secondary tumors (Tables 1–3). The compound is about three orders of magnitude less potent at inhibiting interstitial collagenase and matrilysin than gelatinase A or B, and we found that the gelatinase activity associated with the tumor tissue, but not normal cecum, could be selectively inhibited by CT1746, ex vivo, on zymograms. However, we do not know what concentrations of CT1746 are actually required to inhibit each of the MMPs in vivo, and the blood levels of CT1746 achieved in the model greatly exceed the concentration required to inhibit pure preparations of all four types of MMPs. It is therefore noteworthy that tumor growth inhibition, analogous to that previously described for broad spectrum inhibitors that effectively inhibit collagenase and matrilysin, is observed [7], but with better apparent survival efficacy achieved with CT1746. We cannot rule out the possibility that
Figure 4. Mean body weight in the three groups of mice orthotopically implanted with human colon tumor Co-3. Same experiment as Figure 1. Body weights were measured with an electrical balance. See text for details.

inhibition of one or more of the newly described MT-MMPs, or an unknown metalloproteinase, may also contribute to the effect.

Given the lack of evidence for any CT1746-associated cytotoxicity we do not believe the tumor response involves cell death, at least not as a direct consequence of the compound. MMPs or related MIs are thought to be responsible for a wide variety of molecular processing events, many of which could be considered to be pro-inflammatory [21, 34]. Although a number of publications have demonstrated the inhibition of TNFα release from inflammatory cells by MMP inhibitors [21, 34 and references therein], the selectivity of CT1746 is such that its IC₅₀ value for TNFα release in vitro is in excess of 10 μM. Furthermore, in vivo studies confirmed that at doses ranging from 7 to 700 mg/kg, CT1746 does not inhibit TNFα release following LPS stimulation in immunocompetent BALB/C mice (unpublished observations). It is therefore most unlikely that CT1746 exerts its effects in tumor models through inhibition of a TNFα-mediated inflammation pathway.

The control of tumor growth and spread described here may be at least in part due to an anti-angiogenic effect. Hydroxamic acid-based MMP inhibitors have been reported to inhibit angiogenesis [9, 10] and evidence that CT1746 can inhibit angiogenesis comes from our observation that when administered under identical conditions to those described here, it inhibited the rate of neovascularization of sponges implanted in the flanks of mice [35 and manuscript in preparation]. The effect is dose-dependent and occurs over a period of at least 25 days following sponge implantation.

A further mechanism via which CT1746 may be exerting its effect may be related to the recently published observation that gelatinase functions to modulate cell attachment, and hence cell migration and invasion [2]. To date, however, this has only been demonstrated for melanoma cells, where gelatinase A activity was shown to have a direct effect on adhesion and spreading.

It has been suggested that cytotoxic chemotherapy has reached the limit of efficacy [11, 36] and that approaches based on cell control, rather than cell kill, are becoming a credible alternative. The prototype compound of this general class is the anti-oestrogen tamoxifen [37], that has activity in breast cancer. More recently, vitamin A acid has demonstrated activity in acute promyelocytic leukemia [38]. MMP inhibitors and other anti-angiogenic agents are currently undergoing evaluation in patients with solid tumors. This study suggests that if tumor...
control, in the absence of tumor cell kill, can be achieved then a survival advantage will accrue.

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