Carboxymethyl Benzylamide Dextran and Tamoxifen Combination Inhibits Tumor Growth and Angiogenesis

Rozita Bagheri-Yarmand, Yamina Hamma-Kourbali, Philippe Bissieres, Jean-François Morère, and Michel Crépin

Laboratoire d’Oncologie et Imagerie des Tumeurs Solides, Faculté de Médecine de Bobigny, Université Paris 13 [Y. H.-K., M. C.]; Laboratoire d’hémato logie [P. B.]; and Clinique Universitaire de cancérologie [J-F. M.], 93017 Bobigny Cedex, France; and Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center-108, Houston, Texas 77030 [R. B.-Y.]

ABSTRACT

We showed previously that a carboxymethyl dextran benzylamide (CMDB7) blocks angiogenesis of MDA-MB-435 carcinoma and its lung metastases in nude mice. In this study, we examined the combination effects of CMDB7 and tamoxifen (TAM) on cell proliferation, tumor growth, and angiogenesis on the MCF-7RAS cells. We showed that CMDB7 and TAM acted in a synergistic manner to inhibit the growth of MCF-7RAS cells, blocking them in G0/G1 phase of the cell cycle. For 7 weeks, the CMDB7- (300 mg/kg/week) and TAM- (20 mg/kg/week) treated groups showed tumor growth inhibition of about 66% and 76%, respectively. Combined treatments with CMDB7 and TAM block the tumor development by 94% and induce a complete regression of 4 of 8 mice. Histological analysis showed markedly less neovascularization (88%) in the tumors treated with a combination of CMDB7 and TAM. This antiangiogenic activity was further demonstrated by direct inhibition of endothelial cell proliferation. Overall, this study points to the potential use of a combination of CMDB7 and TAM to inhibit tumor angiogenesis that can prevent tumor progression.

INTRODUCTION

The antiestrogen TAM is being used extensively in hormonal therapy of breast cancer. TAM activity was originally attributed to its ability to inhibit estrogen mitogenic effects, but other mechanisms have been proposed, including down-regulation of oncogenes such as c-myc and c-erbB2 (1), inhibition of protein kinase C (2), and regulation of the cell cycle machinery (3). In addition, TAM has been shown to cause tumor necrosis and regression via inhibition of angiogenesis in MCF-7 breast tumor xenografts (4). Nevertheless, this treatment strategy has had limited success in controlling breast cancer progression because of the transition of breast cancer cells from an estrogen-sensitive to estrogen-insensitive variety in later stages of disease when the tumor becomes more aggressive (5) and highly angiogenic (6). For these patients, the inhibition of angiogenesis with specific angioinhibitory and antimetastatic drugs, such as the dextran derivative CMDB7, in combination with TAM may be a promising new therapeutic strategy.

CMDB7 (7), a noncytotoxic substituted dextran, displays an in vitro growth-inhibitory activity on breast tumoral cells. Growth inhibition was associated with an accumulation of G0/G1 phase cells (7, 8). CMDB7 specifically inhibited the mitogenic effect and receptor binding of angiogenic factors by forming complexes with them (8, 9) and preventing endothelial cell proliferation and migration (10). In vivo studies in nude mice demonstrated that growth of MCF-7RAS (8) and H9 (hst/FGF-4 gene-transfected HBL100 human breast epithelial cell line; Ref. 10) tumors was blocked by CMDB7 treatment, whereas histological analysis showed much less neovascularization in H9-treated tumors. In addition, by inhibiting angiogenesis, CMDB7 is able to inhibit by 88% the incidence of lung micrometastasis from MDA-MB-435 breast carcinoma implanted in the mammary fad pad of nude mice (11). On the basis of this mode of action, we have tested the efficacy of CMDB7 in combination with TAM on tumor growth and tumor angiogenesis in MCF-7RAS human xenografts in nude mice as a mammary tumor model. The MCF-7RAS cells, which express ERs and respond to E2 stimulation, can form tumors in nude mice in the absence of E2 supplementation (12).

MATERIALS AND METHODS

Dextran Derivative Preparation. A water-soluble dextran derivative (CMDB7) was prepared as described previously (11). The chemical composition was determined by acidimetric titration and elementary analysis of nitrogen (dextran, 0%; carboxymethyl, 70%; benzylamide, 30%; mass apparent molecular weight, 80,000 g/mol).

Cell Lines and Cell Cultures. The human malignant breast cell line MCF-7RAS was kindly provided by Dr. C. Sommers (Lombardi Cancer Center, Washington, DC) at passage 7 and was used at approximately passage 30. Human umbilical vein endothelial cells HUVEC were purchased from the American Type Culture Collection (Rockville, MD) at the second passage and were used at approximately passage 6. MCF-7RAS cells were routinely grown in DMEM (Life Tech-
nologies, Inc., Gaithersburg, MD) and HUVEC cells in M199 (Life Technologies, Inc.). Both media were supplemented with 10% FCS, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin, and 50 mg/ml streptomycin (all of these were obtained from Life Technologies, Inc.) at 37°C in a 5% CO₂-humidified atmosphere.

Cell Proliferation. Cells were seeded at a density of 2 x 10⁴ cells/well in Flacon 24-well tissue culture plates (Polylabo) in 10% FCS/DMEM (MCF-7RAS) or M199 (HUVEC) and allowed to adhere to the plastic for 24 h. They were washed with DMEM and incubated with the indicated concentrations of CMDB7 or TAM or, in combination, diluted in the medium containing 1% FCS. After 72 h, cells were washed with PBS and counted using a Coulter counter (Coultronics, Margency, France). Each experiment was carried out in triplicate.

Isobologram Analysis of Interactions between CMDB7 and TAM. The isobologram analysis evaluates drug interactions over a given range of drug concentrations and molar ratios (13). Additionally, isobologram categorizes the degree of interaction between two drugs, regardless of whether the drugs act through similar or different mechanisms (13). The interaction between the two drugs administered is defined as “simple addition” when the effect of the combined drugs is the sum of the effects of the drugs administered separately. “Synergism” is defined in this instance as a mixture of drug I and drug II, giving a greater effect than the sum of their individual effects.

DNA Flow Cytometry. MCF-7RAS cells (10⁶) were plated at 10% FCS/DMEM in T-75 tissue culture flasks. After 24 h, cells were washed with DMEM and incubated with the indicated concentrations of CMDB7 or TAM or in combination, diluted in the medium containing 1% FCS. After 72 h, cells were incubated with BrdUrd (PharMingen, San Diego, CA) for 4 h. Then cells were washed with PBS and fixed with 70% ethanol. Incorporated BrdUrd was revealed by using anti-BrdUrd-MAB conjugated with FITC (PharMingen). Cells were centrifuged and resuspended in a staining solution containing 50 µg/ml propidium iodide (PharMingen) for 10 min. Double-stained cells were analyzed by FACScan (Coulter Epics Laser, CA).

Animals Studies. Female athymic nude mice (nu/nu), 4 weeks old (n = 50), were obtained from Janvier Laboratory (Le-Genest-st-Isle, France). Animals and cells were prepared as described previously (11). Cells (5 x 10⁶) were inoculated s.c. in the right flank of nude mice. This protocol resulted in the apparition of 70% single s.c. palpable tumors 4 weeks after inoculation. Animals bearing MCF-7RAS xenografts (32 mice) were arbitrarily placed in control, CMDB7, TAM, or combined TAM and CMDB7 groups. Mice were treated for 7 weeks by s.c. injection of 0.1 ml of PBS alone (control) or containing 300 mg/kg/week (CMDB7), 20 mg/kg/week (TAM), or in combination. Tumors were measured along two major axes with calipers. Tumor volume (mm³) was calculated as follows:

\[ V = \frac{4}{3} \pi R_1^2 R_2 \]

where \( R_1 \) is radius 1, \( R_2 \) is radius 2, and \( R_1 < R_2 \).

Tissue Preparation and Immunohistochemical Analysis. Immediately after surgical resection, primary tumor specimens were weighed and cut into small pieces. Fragments were fixed with 4% formalin, processed to paraffin in the usual way, and 4-µm sections were stained with H&E. Endothelial cells were specifically stained with GSL-1 lectin (Vector Laboratories, Burlingame, CA) as described previously (11).

Image Analysis. For each GSL-1-labeled section of control-, CMDB7-, TAM-, or combination-treated tumors, five fields containing exclusively viable tumoral cells, as indicated by the hematoxylin stain, were selected randomly for analysis. Image analysis was performed on a Power Macintosh computer 8500/120 using the public domain NIH program (developed at NIH and available on the Internet⁴). The endothelial cell area in each section was determined as described previously (11). The percentage area of endothelial cells was then calculated as the ratio of the labeled area:the total viewed area x 100. These values were then averaged for untreated (control) and treated (CMDB7, TAM, or combination CMDB7 and TAM) tumors.

Statistical Analysis. The results are presented as means ± SE. Multiple statistical comparisons were performed using ANOVA and the Mann-Whitney U tests in a multivariate linear model.

RESULTS

Dose Response of CMDB7 and TAM on MCF-7RAS and HUVEC Cell Proliferation. Initial experiments were performed to determine the range of drug concentrations that would elicit partial responses on the MCF-7RAS and HUVEC cell growth inhibition. To determine the optimal concentration for each drug, MCF-7RAS and HUVEC cells were incubated with 0.01–20 µM CMDB7 or with 0.01–3 µM TAM. Fig. 1, A and B, shows that MCF-7RAS cells were significantly inhibited by both CMDB7 and TAM in a dose-dependent manner and that HUVEC cells were more inhibited by CMDB7 than TAM at pharmacologically used concentrations. The percentages of MCF-7RAS growth inhibition by 10 and 20 µM CMDB7 were 41 ± 4 and 68 ± 3, respectively (Fig. 1A). The same concentrations of CMDB7 inhibited the HUVEC growth by 64 ± 2 and 71 ± 3, respectively (Fig. 1A). Whereas 1 and 3 µM TAM inhibited the MCF-7RAS growth by 38 ± 2 and 62 ± 3, respectively (Fig. 1B), 3 µM TAM inhibited the HUVEC growth by only 21 ± 3 (Fig. 1B).

Isobologram Analysis of the Effects of Combination Treatment with CMDB7 and TAM. To assess the effect of the CMDB7 and TAM combination on the cell growth, MCF-7RAS and HUVEC cells were incubated with various concentrations of CMDB7 in the presence or absence of 0.01 µM TAM (Fig. 2A), 0.1 µM TAM (Fig. 2B), and 1 µM TAM (Fig. 2C). The combination of CMDB7 and TAM resulted in a more effective growth suppression than either CMDB7 or TAM alone (Fig. 2). By comparing results from Fig. 1 with the latter results, we can conclude that this inhibition is statistically significant (P < 0.05) at all of the concentrations for both MCF-7RAS and HUVEC cells.

The data from Fig. 2, showing that CMDB7 and TAM compounds interfered most effectively at lower concentrations

of CMDB7, were used to design an isobologram experiment to determine whether the two drugs are capable of acting synergistically. Fig. 2D shows an isobologram for the interaction of CMDB7 and TAM on the MCF-7RAS cell line. The ID$_{50}$ and 95% confidence limits for CMDB7 alone (15 ± 1 µM) and for TAM alone (2 ± 0.1 µM) are shown, connected by diagonal lines (Fig. 2D). The ID$_{50}$ and 95% confidence limits for the combined drugs were plotted along a line beginning at the origin and extending along a fixed drug ratio of 10:1, where the dose of TAM is plotted on the X axis and the dose of CMDB7 is plotted on the Y axis. The ID$_{50}$ for the combined drugs (7 ± 0.2 µM CMDB7 and 0.01 ± 0.001 µM TAM; Fig. 2D) falls below the line connecting the single-drug ID$_{50}$, indicating that CMDB7 and TAM interact synergistically at the given concentration to inhibit the MCF-7RAS cell line.

The isobologram for the interaction between CMDB7 and TAM on the HUVEC cells shows that the ID$_{20}$ (ID$_{20}$, because 21% was the maximum percentage of inhibition obtained by TAM at used concentrations) for TAM by itself was 3 ± 0.2 µM and for CMDB7 by itself was 2 ± 0.1 µM (Fig. 2E). When the two drugs were tested in combination, the ID$_{20}$ for TAM was 1 ± 0.1 µM and for CMDB7 was 0.1 ± 0.01 µM (Fig. 2E). When plotted along the 10:1 fixed-drug ratio line, the combination of the two drugs again falls below the line connecting the single-drug ID$_{50}$, indicating that CMDB7 and TAM act synergistically to inhibit the growth of HUVEC cells at the given concentrations.

Cell Cycle Analysis of the Combined Treatment of CMDB7 and TAM. Flow cytometry profiles revealed that treatment with increasing doses of CMDB7 or TAM led to a dose-dependent shift in the percentage of cells with a G$_0$/G$_1$-like DNA content (Table 1). Consistent with the cell growth studies, a combination of both agents caused a more striking shift to G$_0$/G$_1$ phase of the cell cycle over that with either drug alone. The CMDB7- and TAM-mediated shift in number of G$_0$/G$_1$ cells appeared to result from a decrease in S-phase cells, whereas the G$_2$-M phase values did not significantly change (Table 1). Results from a representative experiment using flow cytometry of propidium iodide-stained cells are shown in Fig. 3.

Synergistic Antitumor Effects of CMDB7 and TAM on MCF-7RAS Human Breast Carcinomas in Nude Mice. Treatment started when the average tumor volume reached 600 mm$^3$, 4 weeks after MCF-7RAS cell injections. Control tumors increased from 600 ± 206 to 6695 ± 1135 mm$^3$ after 7 weeks. Overall, continuous treatment for 7 weeks with CMDB7 (300 mg/kg/week) or TAM (20 mg/kg/week) as single agents inhibited the growth of MCF-7RAS xenografts by 66% and 76% ($P < 0.001$), respectively (Fig. 4). Combined treatment of MCF-7RAS tumor-bearing mice with CMDB7 and TAM blocked the tumor development by 94% (Fig. 4), consistent with in vitro studies. Moreover, in four mice, tumors were completely suppressed.

Synergistic Anti-Angiogenic Effects of CMDB7 and TAM on MCF-7RAS Human Breast Carcinomas in Nude Mice. GSL-1 selectively labeled endothelial cells (Fig. 5, A–D) and thus enabled the relative density of endothelial cells (percentage of area occupied by endothelial cells) to be determined based on the image analysis of the label detection. The mean percentages of endothelial cell area in viable fields of CMDB7- and TAM-treated tumors were inhibited by 62% (40 fields in eight tumors) and 56% (40 fields in eight tumors), respectively, as compared with the control tumors (40 fields in eight tumors). Combined treatment with CMDB7 (300 mg/kg/week) and TAM (20 mg/kg/week) inhibited angiogenesis by 88% (30 fields in four tumors, because four tumors were completely suppressed). The endothelial cell densities in all of the treated groups were significantly lower ($P < 0.001$).

DISCUSSION

In the present study, both CMDB7 and TAM showed antitumor activity against MCF-7RAS tumors xenografted in...
nude mice. The tumor growth inhibition by CMDB7 is visible after 1 week of treatment and increases from 50% to 66% after 7 weeks. Although TAM inhibited tumor growth by 76%. The combination of CMDB7 and TAM inhibited tumor growth by about 94% demonstrating a synergic effect between the two agents. In addition, four tumors were completely regressed by the combination of TAM and CMDB7 treatment.

One mechanism through which CMDB7 and TAM inhibited tumor growth might be the inhibiting of angiogenesis. The role of angiogenesis in tumor progression and cellular invasive-
ness is well documented in clinical and experimental studies (14). In addition, breast cancer is known to be highly vascularized, and several studies (4, 6) have attempted to correlate the degree of neovascularization with prognosis. In the CMDB7- and TAM-treated tumors, a reduction of 62% and 56%, respectively, in the number of capillary vessels occurred in viable tumor regions, indicating that this treatment attenuated the rate of neovascularization but did not completely block the initial activation of angiogenesis. Combination of CMDB7 and TAM treatment demonstrated a more marked antiangiogenic effect with 88% inhibition of tumor angiogenesis. The evident antangiogenic effect of CMDB7 was reported in other breast tumor models such as xenografted MDA-MB-435 (11) and HH9 human breast cells (10). In MDA-MB-435 breast carcinoma xenografted in mammary fat pad, the inhibition of angiogenesis prevented the appearance of distant metastasis in the lung of nude mice (11). Concerning TAM, several studies (15) have shown that TAM also inhibited angiogenesis by a mechanism independent of ER content.

The explanation for the in vivo efficacy of these drugs can be derived from the in vitro experiments. Our studies demonstrated that CMDB7 inhibited endothelial cell proliferation in a dose-dependent manner. These results are in agreement with previous studies (10) that demonstrated that CMDB7 inhibited the two key steps in the angiogenesis process, i.e., the in vitro migration and proliferation of endothelial cells, without acting on cell viability. TAM, used at pharmacological concentrations, weakly affected the in vitro growth of endothelial cells as compared with CMDB7. However, simultaneous administration of CMDB7 and TAM displayed a significant synergistic inhibitory effect on the growth proliferation of endothelial cells.

Table 1  Cell cycle analysis of MCF-7RAS cells treated with TAM and CMDB7

<table>
<thead>
<tr>
<th>Phases [TAM (µM)/CMDB7 (µM)]</th>
<th>G_0/G_1</th>
<th>S</th>
<th>G_2/M</th>
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* CMDB7 and TAM combined treatment was significantly different (P < 0.05) versus CMDB7 or TAM separate treatment.
potentiation of antiangiogenic effects by the association of CMDB7 and TAM may be partially responsible for mediation of antitumor effects, in addition to the direct effect on MCF-7RAS tumor cell proliferation. This antiproliferative effect was dose-dependent and significant already at 1 μM for CMDB7 and 0.1 μM for TAM. The combination of CMDB7 and TAM resulted in a more effective growth suppression than either CMDB7 or TAM alone. Inhibitions were statistically significant (P < 0.05) and more marked at very low doses of CMDB7 and TAM (0.001 μM and 0.01 μM, respectively).

The antiproliferative effect of CMDB7 and TAM was supported by flow cytometry analysis. This analysis demonstrated that CMDB7 and TAM decreased the percentage of MCF-7RAS cells in the S-phase with preferential blocking in the G0/G1 phase. Association of CMDB7 and TAM increased the number of cells blocked in G0/G1 phase of the cell cycle. These results are in agreement with our previous studies (7, 8), which demonstrated that the CMDB7 growth inhibition was associated with a decrease in the proportion of S-phase cells and an accumulation of G0/G1 phase cells. In addition, the antiproliferative effects of TAM have been reported (16–18) to result from its ability to inhibit cyclin-dependent kinases, inducing a cell cycle arrest at the G0/G1 checkpoint.

These results show that CMDB7 is more potent for HUVEC cells and that TAM is more potent for MCF-7RAS cells. TAM-inhibited ER-mediated activities are partially responsible for the antiproliferative effects of TAM. So, ER+ MCF-7RAS cells are more sensitive for TAM than HUVEC cells, which do not express ER. Furthermore, TAM has been reported to inhibit embryonic angiogenesis in the chicken chorioallantoic membrane by a mechanism independent of E2 concentration or ER content (15). Protein kinase C, important in the signal transduction cascade for endothelial cell growth, is inhibited by TAM (4). On the other hand, CMDB7 acts via the disruption of cytokine effects, resulting in the blocking of the cell transduction cascade. So, an alternative hypothesis for the synergistic effect between the two drugs could be dependent on modulation of the signal transduction cascade.

CMDB7 exerts its antiproliferative and antiangiogenic activity via the disruption of cytokine effects. We have shown previously (8, 9) that CMDB7 appears to act as an inhibitor of the autocrine and paracrine growth factor by inhibiting the activities of conditioned medium and purified growth factors such as FGF2, TGFβ, and platelet-derived growth factor-BB. The affinity electrophoresis demonstrated that CMDB7 functions by interacting directly with growth factors (FGF2, TGFβ, and platelet-derived growth factor-BB). It is well established that TAM can exert its growth-inhibitory effects in human breast cancer cells by antagonizing the ER stimulation of the cell cycle progression (19). In addition to these ER-mediated effects, TAM has also been found to act on several other targets implicated in breast cancer progression such as modulation of growth factor synthesis like TGFα, TGFβ, and FGF-2 (20). Thus, CMDB7 and TAM may synergistically inhibit angiogenesis and tumor growth through modulation of growth factor secretion by TAM and through inhibition of autocrine and paracrine growth factor production.
paracrine growth factors effects by CMDDB7. The features of each pathway likely plays a role in the more stringent inhibition of tumor growth and cell proliferation observed with both CMDDB7 and TAM.

In conclusion, this study suggests that the combination of CMDDB7 and TAM is capable of producing a synergistic effect on the inhibition of angiogenesis and tumor growth and then can prevent tumor progression. In principle, this treatment could be exploited to circumvent acquired drug resistance in estrogen-insensitive and in highly angiogenic breast cancer. Lower doses are proposed for circumventing TAM resistance. Actually, isobologram analysis of combination effects showed that synergism observed between CMDDB7 and TAM on the proliferation of MCF-7RAS and HUVEC cells was more marked at low concentrations of CMDDB7 and TAM. In any case, our findings suggest potential usefulness in combining the two nontoxic agents for the treatment of malignant diseases.

REFERENCES


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