Enhanced Antitumor Activity of Combination Radioimmunotherapy (\textsuperscript{131}I-labeled Monoclonal Antibody A33) with Chemotherapy (Fluorouracil)\textsuperscript{1}

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ABSTRACT

Monoclonal antibody (mAb) A33 reacts with an antigen expressed by >95% of colon cancer and normal colon epithelial cells. An earlier Phase I trial of \textsuperscript{131}I-labeled mAb A33 (\textsuperscript{131}I-mAb A33) demonstrated bone marrow suppression as the dose-limiting toxicity, and although modest antitumor effects were seen, no normal colon toxicity was observed. In this study, a nude mouse model was used to test whether combinations of low-dose \textsuperscript{131}I-mAb A33 (0.1 mCi) and chemotherapy [5-fluorouracil (5-FU) or 5-FU + leucovorin, doxorubicin, or carbustimine] enhance the antitumor effects, compared to \textsuperscript{131}I-mAb A33 alone or either drug regimen alone. 5-FU was administered either at 30 mg/kg/day for 5 days or at 75 mg/kg/day on days 1 and 5. In assessing the reduction in tumor volumes over the first 28 days of the experiment, 5-FU treatment (with or without leucovorin) in combination with \textsuperscript{131}I-mAb A33 showed a statistically significant additive antitumor effect compared to \textsuperscript{131}I-mAb A33 alone or to chemotherapy alone. When long-term survival was used as an end point, 38% of the mice treated with 5-FU and \textsuperscript{131}I-mAb A33 were disease free at 276 days compared to none from any other group, suggesting a synergistic effect. These data indicate that Phase II clinical trials combining radiolabeled antibody therapy with 5-FU-based treatments are warranted.

INTRODUCTION

Cancer of the colon and rectum is the second most common cancer in the United States (1). Surgery is the cornerstone of treatment, but surgery alone has a high recurrence rate even in the treatment of primary, localized disease. Only lately have adjuvant chemotherapy regimens for colon cancer, or adjuvant chemoradiotherapy regimens for rectal cancer, shown an impact on survival (2–6).

5-FU,\textsuperscript{3} the mainstay of treatment, blocks the activity of thymidylate synthetase and/or is activated to fluoro-UTP and incorporated into the RNA. However, the efficacy rate of 5-FU is limited (7–9). Recently, many attempts have been made to increase the antitumor activity of fluoropyrimidine using biochemical modulators. LV is one such modulator, and its mode of action is considered to be an increase in thymidylate synthetase inhibition through formation of a ternary complex of 5,10-methylene tetrahydrofolate from LV, thymidylate synthetase, and fluorooracil (10–12).

Whether the strategy of 5-FU modulation by LV is clinically significant remains unclear. Some Phase III clinical trials have shown that 5-FU + LV provides a significantly higher response rate than 5-FU alone (13–16), and a recently published multicenter, pooled analysis showed significant survival benefit for adjuvant 5-FU + LV treatment after colon resection (17). However, other studies have not shown a significant benefit (18, 19). Even under these improved conditions, the response rate is at best only 35%. Therefore, new therapeutic approaches are needed to treat this type of cancer.

Another approach to enhance the efficacy of 5-FU is to take advantage of the additive (20, 21) or even synergistic effect of the combination of 5-FU and radiation (22–25), which is believed to account in part for better control of local recurrences of rectal cancer. In primary or advanced colon cancer and advanced rectal cancer, external beam radiation therapy is not applicable for technical reasons and because of adjacent radiosensitive organs. Radiolabeled mAbs targeting colon cancer might be able to deliver the needed radiation dose precisely and without major toxicity. A Phase II \textsuperscript{131}I-mAb A33 therapy trial demonstrated bone marrow as the dose-limiting organ toxicity (26). Due to the localization of radiolabeled antibody to normal colon, gastrointestinal toxicity was examined closely and was found to be minimal. Antitumor effects were observed in 5 of 23 assessable patients, despite the fact that only a single dose could be administered due to development of an antimouse immunoglobulin response (26). To determine toxicity, treatment schedule, and therapeutic efficacy of a combined approach, different combinations of low-dose (0.1 mCi) \textsuperscript{131}I-mAb A33 and chemotherapeutic agents were evaluated in a nude-mouse, human colon cancer-xenograft model. Because the radiation dose delivered to solid tumors by radiolabeled antibodies is low in clinical studies (27), we selected a subtherapeutic dose of \textsuperscript{131}I-mAb A33 for this study to search for enhanced antitumor effects at low radiation dosages.

MATERIALS AND METHODS

Cell Lines. Human colon carcinoma cell line SW1222 was obtained from the cell bank of the Ludwig Institute for Cancer Research at Memorial Sloan-Kettering Cancer Center and maintained in Eagle’s MEM containing 1% nonessential amino acids and supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 \mu g/ml streptomycin. Cells were cultured in a 37°C incubator containing 5% CO\textsubscript{2}. The cells were harvested using 0.1% trypsin and 0.02 EDTA (Life Technologies, Inc., Grand Island, NY).

Human Tumor Xenografts. Four- to 6-week old female Swiss (nu/nu) athymic mice (20–25 g body weight) were obtained from the Memorial Sloan-Kettering Cancer Center nude mouse facility and injected i.m. with 10 x 10\textsuperscript{6} cells in 250 \mu l PBS into the left thigh muscle. After 1 week, mice bearing tumors weighing approximately 100–500 mg were selected. Mice were anesthetized with 0.5 ml Avertin (2,2,2-tribromoethanol, 97%; Pfaltz and Bauer, Waterbury, CT) by i.p. injection.

Labeling of mAbs. Iodination of mAbs was carried out using the chloramine T method. mAb A33 and mAb FB5, an IgG2a against human neovascular endothelium used as an isotype control, were mixed with the requisite quantity of 131I (1 mg/20 mCi). Two hundred \mu l of a freshly prepared solution of 2 mg/ml chloramine T (in 150 mM sterile phosphate buffer at pH 7.4) were added to the mixture. After 1 min, the reaction was quenched by the addition of 200 \mu l of a freshly prepared solution of 10 mg/ml sodium metabisulfite. Labeled protein was separated from the free iodine on a Sephadex G25 column (Pharmacia), saturated with 0.5% BSA/PBS. Antibody fractions with peak radioactivity were pooled, and aliquots of the pooled fractions were tested for the percentage of precipitable protein-bound 131I by the trichloroacetic acid

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\textsuperscript{3}The abbreviations used are: 5-FU, 5-fluorouracil; LV, leucovorin; mAb, monoclonal antibody; \textsuperscript{131}I-mAb A33, \textsuperscript{131}I-labeled mAb A33, \textsuperscript{131}I-mAb FB5, \textsuperscript{131}I-labeled mAb FB5; MTD, maximum tolerated dose; AUC, area under the fitted growth curve; % IDg, % of injected dose/g; MTS, mean tumor size.
precipitation method. The amount of trichloroacetic acid-precipitable protein-bound $^{131}I$ was 97–99%.

Immunoactivity for mAb A33 was determined as described previously (28) by adding 0.1 μCi of $^{131}I$-mAb A33 to 15 x 10^6 antigen-positive cell pellets. To determine background cpm, cell pellets were pretreated with >100 × excess unlabeled mAb A33 prior to addition of $^{131}I$-mAb A33. Immunoactivity was calculated by subtracting background cpm from $^{131}I$-mAb A33 cpm bound to the cell pellet, after washing twice in PBS and dividing the remaining fraction by the total cpm added. Mean immunoactivity was 34%. A single dose of 0.1 μCi of $^{131}I$-mAb A33 or $^{131}I$-mAb FB5 in 0.2 ml of PBS was injected i.v. into the retro-orbital plexus.

Formulation and Administration of Drugs. 5-FU (50 mg/ml; SoloPak Laboratories, Franklin Park, IL) was obtained as a pharmaceutical preparation and diluted to a concentration of 5 mg/ml or 10 mg/ml in sterile 0.9% saline. It was injected i.p. either at 30 mg/kg/day for 5 days or at 75 mg/kg/day on days 1 and 5. The injected volume ranged from 0.12 to 0.19 ml.

LV (Burroughs Wellcome Co., Research Triangle Park, NC) was dissolved in 0.9% sterile saline at a concentration of 10 mg/ml. Mice treated directly received 90 mg/kg LV i.p. 2 h prior to 5-FU. The injected volume was 0.18 ml/20 g body weight.

Doxorubicin (Sigma Chemical Company, St. Louis, MO) was given i.p. as a single dose of 10 mg/kg. Carmustine (Bristol Myers Squibb, Princeton, NJ) was injected i.p. as a single dose of 30 mg/kg. Both drugs were dissolved in PBS sufficient to yield a volume that permitted administration in a dose of 0.1 ml/10 g body weight and injected on day 1.

The doses used were selected from the literature and first tested in tumor-bearing animals in preliminary toxicity studies to determine the MTD.

Tumor Therapy. Mice were divided into groups of six to eight animals. Toxicity was determined by measuring change in body weight. All animals were weighed at the start of the experiments and at 8-day intervals. Tumor size was determined by digital caliper measurement. Two perpendicular diameters were used to calculate tumor volumes (29). Measurements were taken immediately before therapy and at 4-day intervals until day 68 or until tumor volume reached 2 cm³ (2 g).

In the first experiment, one group of mice each received 0.1 μCi of either $^{131}I$-mAb A33 or control $^{131}I$-mAb FB5, 5-FU daily × 5, or 5-FU on days 1 and 5. Additional groups were treated either with a combination regimen of 5-FU daily × 5 + $^{131}I$-mAb A33, 5-FU on days 1 and 5 + $^{131}I$-mAb A33, 5-FU daily × 5 + $^{131}I$-mAb FB5, or 5-FU on days 1 and 5 + $^{131}I$-mAb FB5. Growth controls consisted of mice receiving no treatment. In the second experiment, the effectiveness of 5-FU + LV + $^{131}I$-mAb A33 was compared with that of $^{131}I$-mAb A33 alone and with 5-FU + LV. Again, an untreated control group was included. In the third experiment, the effect of 5-FU + LV on tumor growth was compared to doxorubicin or carmustine, each with or without $^{131}I$-mAb A33.

Statistical Methods. Within each treatment group, the growth curves of tumors were fitted with a linear regression model, up to second order, of time (day) after logarithmic transformation. The model can be displayed as:

$$
\log(\text{tumor volume}) = A + B \times \text{day} + C \times \text{day}^2
$$

The regression coefficients A, B, and C were estimated using a generalized estimation equation (30). In comparing tumor growth between groups, the AUC (measuring tumor volume accumulated over time) from day 0 to day 28 was the endpoint of comparisons, with AUC defined as:

$$
\text{AUC} = 28A + 28B^2 + 28C^3
$$

where A, B, and C are the estimated regression coefficients.

A smaller AUC compared to the control group AUC indicates a treatment effect on tumor growth inhibition. We assumed that the effect of a regimen composed of two agents is equivalent to the sum of the separate effects of the two agents plus the synergistic effect, if it exists. As an analogue to the test of interaction in the two-way ANOVA, the synergy between agents A and B was tested by applying a $Z$ test on the difference between (a) AUC of treatment A and B + AUC of control and (b) AUC of treatment A + AUC of treatment B. If the tests of synergy were not significant, the tests of main effects were pooled over different treatment combinations using the $Z$ test. For observations beyond 28 days, the proportion of long-term survival between groups was compared using Fisher’s exact test. Three separate experiments were performed. Only the treatment groups within the same experiment were compared to each other. For the comparisons within an experiment, Type I errors were adjusted using Bonferroni’s method.

RESULTS

A dose-response curve for $^{131}I$-mAb A33 was established previously 4; the lowest dose of $^{131}I$-mAb A33 giving an antitumor effect was 0.1 μCi. This dose was tested again and confirmed to be the lowest dose producing the smallest measurable tumor-suppressive effect. It was therefore selected for our studies (see below).

The mean AUCs from day 0 to day 28 for each group are shown in Table 1. Many of the AUCs are negative due to logarithmic transformation of small values (<1). In a preliminary experiment, 5-FU was tested at doses ranging from 15 to 30 mg/kg daily × 5 or at 40–75 mg/kg on days 1 and 5 (data not shown), but the maximum dose given by daily injection or on days 1 and 5 achieved only a minimum antitumor effect (Fig. 1).

In an earlier study, unlabeled mAb A33 had shown no demonstrable effect on the growth of established tumors in this model. 4 Likewise, the isotype-matched control $^{131}I$-mAb FB5 showed no significant effect on tumor growth when compared to untreated controls (Fig. 2).

The combination of 5-FU given daily × 5 or on days 1 and 5 + $^{131}I$-mAb FB5 at 0.1 μCi showed no increased tumor-suppressive effects compared to 5-FU alone (Fig. 3). The combination of 5-FU and $^{131}I$-mAb A33 resulted in significantly higher antitumor activity (Fig. 4). In the groups of mice that were treated either with 5-FU daily × 5 + $^{131}I$-mAb A33 or with 5-FU on days 1 and 5 + $^{131}I$-mAb A33, one xenograft in both groups escaped the therapy relatively early. Three of the eight mice in each group developed minimal tumor growth 54–60 days after treatment. The other four animals in each group had no evidence of tumor growth until 80–84 days after treatment, when one of the four mice in each group had a recurrence. The remaining three animals (38%) in both groups had no recurrence as late as 276 days following treatment. Thus, statistical analysis showed that long-term, disease-free survival for both of these com-

<table>
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<th>Treatment</th>
<th>AUC (ln (cm³) X day)</th>
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<tr>
<td>Experiment 1</td>
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<tr>
<td>$^{131}I$-mAb A33</td>
<td>−48.3</td>
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<tr>
<td>$^{131}I$-mAb FB5</td>
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<td>1.9</td>
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<tr>
<td>Control</td>
<td>26.4</td>
<td>5.3</td>
</tr>
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4 E. Barendswaard, S. Welt, F. Daghghian, A. Scott, M. Graham, and L. J. Old, unpublished observations.
2.5 combination therapies was significantly different from that of 5-FU alone or 131I-mAb A33 alone (Fisher's exact test, \( P < 0.05 \)). A synergistic antitumor effect is suggested when the groups are compared for survival over the entire observation period (276 days).

A similar set of experiments comparing the combination of 5-FU + LV and the addition of 131I-mAb A33 showed that chemotherapy and radioimmunotherapy have significantly more antitumor effects (Fig. 5), whereas the addition of 131I-mAb FB5 to 5-FU + LV did not increase antitumor activity significantly (Fig. 5).

To determine whether combination therapy had additive or synergistic effect on reduction of tumor size, differences in tumor volume were analyzed. When the tumor weight of mice reached 2 g, the animals were sacrificed. Therefore, the analysis was carried out only until tumor volumes reached 2 cm³. No statistical differences were observed in the analysis evaluating synergistic antitumor effects for the combination of 5-FU given daily \( \times 5 \) or on days 1 and 5 + 131I-mAb A33 and either treatment alone for days 1–28. However, the two different treatment modalities had additive antitumor activity.

To determine whether the enhanced tumor-suppressive effect of 5-FU + 131I-mAb A33 with or without LV might be common to chemotherapeutic agents in general or specific to certain drugs, we tested combinations of doxorubicin + 131I-mAb A33 and carmustine + 131I-mAb A33. The addition of these two chemotherapeutic agents did not improve the antitumor activity of 131I-mAb A33 compared to radiolabeled antibody alone (data not shown).

Two deaths unrelated to tumor growth occurred during treatment: one animal of eight (12.5%) in the group treated with 5-FU daily \( \times 5 \) + 131I-mAb FB5 and one of six (16.5%) in the group treated with doxorubicin + 131I-mAb FB5. Minor weight loss was observed in several animals treated with 131I-mAb FB5 and with doxorubicin. No signs of toxicity were seen in animals treated with 5-FU and LV as single agents and with any combination plus 131I-mAb A33.

DISCUSSION

Adjuvant chemotherapy, radiation, and immunotherapy have shown some usefulness in the treatment of primary colorectal carcinoma and its more advanced stages (31). However, agents with better antitumor activity need to be identified if significant progress is to be made.

Recent studies have shown a significant survival benefit (6, 32) for patients treated with chemotherapy and external beam radiation after curative surgery for rectal cancer. The underlying cause for this phenomenon is thought to be radiosensitization. In vitro and in vivo studies of the combined effects of 5-FU and radiation (21, 33–37) have suggested that any increase of effect over radiation alone is not due to inhibition of repair of sublethal or potentially lethal damage. The mode of interaction of the drug with radiation remains unclear. In addition, the sequencing of 5-FU and radiation treatments may be a crucial parameter. It has been suggested that synergism could be achieved only by 5-FU infusions, with brief injection schedules yielding additive effects (35). Other investigators (21, 38) found only additive antitumor effects without evidence for sequence dependence. Nevertheless, clinical trials have suggested a greater than expected local control rate with 5-FU chemoradiation for both squamous cell carcinomas and adenocarcinomas (39).

In colon cancer, external beam radiation has its limits for technical reasons and because of toxicity to adjacent organs. Lacking these drawbacks, radiolabeled antibodies might be an alternative to deliver...
radiation therapy in colon cancer. The realistic evaluation of radiolabeled antibodies as radiotherapeutic agents, however, depends on accurate dosimetry calculations. This is key information for radioimmunotherapy trials in solid tumors that will require continuous monitoring of isotope levels in essential tissues for the length of time the isotope is retained. Positron emission tomography analysis offers an approach to obtaining this information.

Another important consideration is microdosimetry, which takes into account the architecture of the tumor and the distribution of antibody. Our earlier studies with mAb A33 have shown that localization was specific when $^{99m}$Tc-albumin was compared to radiolabeled mAb A33 as a marker for tumor blood pool uptake, and when $^{125I}$labeled isotype-matched control antibody was compared to $^{131I}$labeled A33 (28). Using autoradiographs, we were able to show that isotope accumulation in tumors corresponded to the antibody binding.

Fig. 3. Effect of nonspecific radioimmunotherapy in combination with chemotherapy on tumor growth. 5-FU daily, n = 6; MTS, 0.344 cm$^3$. 5-FU daily + $^{131I}$-mAb FB5, n = 8; MTS, 0.342 cm$^3$. 5-FU bolus, n = 6; MTS, 0.369 cm$^3$. 5-FU bolus + $^{131I}$-mAb FB5, n = 8; MTS, 0.349 cm$^3$.

Fig. 4. Effect of addition of specific $^{131I}$-mAb A33 radioimmunotherapy to chemotherapy on tumor growth. $^{131I}$-mAb A33, n = 8; MTS, 0.329 cm$^3$. 5-FU daily, n = 6; MTS, 0.344 cm$^3$. 5-FU daily + $^{131I}$-mAb A33, n = 8; MTS, 0.392 cm$^3$. 5-FU bolus, n = 6; MTS, 0.369 cm$^3$. 5-FU bolus + $^{131I}$-mAb A33, n = 8; MTS, 0.376 cm$^3$. 5-FU 30 mg/kg daily x 5 + $^{131I}$-mAb A33, n = 8; MTS, 0.392 cm$^3$.
specifically to the tumor cells while the surrounding stromal cells and vasculature did not concentrate the isotope. Thus, dosimetry calculations have provided only estimates of radiation doses delivered to tumor cells. Single-cell dosimetry with long-range β-emitters such as 131I provide an even greater challenge. In general, our present knowledge is based on external and biopsy-based measurements; however, tumor tissue doses reported to be absorbed are only a fraction of what is needed to eradicate tumors (27).

Human cancer xenograft models in nude mice have proved to be valuable in testing the therapeutic potential of new reagents such as radiolabeled antibodies. However, when evaluating the results of these models, an important consideration is the limitation in predicting efficacy in human studies. One major consideration is that antibody uptake by the tumor can be 3000-fold higher in the mouse model than in patient studies (30% vs. 0.01% ID/g tumor). This is partially compensated for by the higher total dose used in humans compared to mice. Because the MTD of 131I in humans is almost 300 times that in mice, this still leaves a 10-fold higher tumor tissue dose in mice than in humans at the MTD. Thus, it is important to select doses for experiments with animal models that are a fraction of the MTD. In general, animal models may in some cases exaggerate the antitumor effect of experimental therapy regimens, as it does with radiolabeled antibodies. However, when this issue has been examined in other therapeutic systems, it was shown that human xenografts in mice seem to retain histology and chemosensitivity of the original tumor (40) and that radiosensitivity of individual tumor cells correlates well with the original tumor (41).

For the studies presented here, we selected a 131I-mAb A33 dose that was 20% of the MTD. On the basis of the % ID/g tumor observed in this animal model, peak 131I uptake corresponds to 0.035 mCi/g. In our clinical studies with 131I-mAb A33, uptake (% ID/g) was measured by biopsy on day 7 or 8 after antibody administration. In dosimetry studies, patients with the highest uptake of 131I-mAb A33 would achieve isotope concentrations of 0.015–0.045 mCi/g at treated at the MTD (75 mCi/m2). Thus, the effects observed in the present study may be correlated directly to the subgroup of patients with the highest radiolocalization indices for 131I-mAb A33. Our results show an additive effect of combination chemotherapy (5-FU or 5-FU + LV) plus low-dose radioimmunotherapy. When survival was used as an end point, a synergistic effect was found, in contrast to earlier findings in a mouse radioimmunotherapy model using an anti-CEA antibody (42). No differences were observed in antitumor effects when the two 5-FU treatment schedules were compared, and both demonstrated enhanced activity when combined with specific radioimmunotherapy. In the clinic, the dose-limiting toxicity for bolus and continuous-infusion 5-FU is different, and the relative lack of hematological toxicity of the continuous-infusion schedule makes this an attractive partner for 131I-labeled antibody-based therapies. Due to the low nontumor radiation dose and specific uptake of radiolabeled antibody, we could not detect any signs of toxicity in our animals. In patients treated with 5-FU/mAb A33 chemoradioimmunotherapy, we might expect enhanced antitumor effects with little change in toxicity, due to the low nonspecific radiation doses delivered to critical tissues manifesting 5-FU toxicity. The enhanced antitumor effects were specific for 5-FU-based treatments as the addition of other chemotherapeutic agents (doxorubicin and Carmustine or even LV) to 131I-mAb A33 had no beneficial effect.

In theory, mAbs can deliver radiation therapy continuously over prolonged periods. Hyperfractionated radiation dosing, in which the rationale is based on growth and on radiation repair patterns of tumor cells, has shown advantages over single doses (43–45). After a single antibody administration, the actual dose rate to the tumor does not simulate prolonged periods. Hyperfractionated radiation dosing, in which the rationale is based on growth and on radiation repair patterns of tumor cells, has shown advantages over single doses (43–45). After a single antibody administration, the actual dose rate to the tumor does not simulate prolonged periods. Hyperfractionated radiation dosing, in which the rationale is based on growth and on radiation repair patterns of tumor cells, has shown advantages over single doses (43–45). After a single antibody administration, the actual dose rate to the tumor does not simulate the half-life effect of the isotope used (e.g., 131I) and the continuous clearance of isotope from the tumor. With the generation and clinical use of humanized antibodies, therapeutic regimens may be developed that will approximate hyperfractionation because the humanized antibody can be given repeatedly.

The question of whether the response to continuous, exponentially decreasing irradiation, which is characteristic of radioimmunotherapy, is biologically equivalent to conventionally fractionated, high-dose rate irradiation remains to be solved. Very few studies have addressed this question (46–53). Our results here have clearly demonstrated an improvement in antitumor effects by chemoradioimmunotherapy in an animal model. This finding should be tested in clinical studies in subsets of patients with advanced disease, who have not yet been treated with 5-FU. We have observed that in a group of heavily pretreated patients, who received chemotherapy after a Phase I radioimmunotherapy trial with 131I-mAb A33, a significant number had major responses (54). These unexpected findings need to be explored further and verified in well-designed clinical studies. Preclinical studies to identify other chemotherapeutic agents with additive or synergistic activity when combined with radiolabeled antibodies should continue, especially with the newer agents identified recently as having activity in colon cancer.
REFERENCES


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