Cancer Chemother Pharmacol (1992) 29: 290–296

Cancer Chemotherapy and Pharmacology

In vitro antineoplastic activity of C7-substituted mitomycin C analogues MC-77 and MC-62 against human breast-cancer cell lines

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Received 3 June 1991/Accepted 9 August 1991

Summary. Mitomycin C (MIT-C) is one of the most potent antineoplastic agents used for the treatment of breast cancer and a wide variety of malignant tumors. However, administration of MIT-C is frequently accompanied by the delayed onset of severe myelosuppression. We have synthesized a new series of MIT-C analogues that are predicted on a structure/function basis to retain cytotoxicity but exhibit decreased toxicity. These new compounds feature a sugar substitution at the N7 position. Using a series of human breast-cancer cell lines growing in vitro, we determined the structure/activity relationship of two independent N_7-substituted spacers displaying the same glucopyranose moiety. *N*-{[(2-acetamide-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)amino]carbonyl} propylmitomycin C (MC-62) contains the sugar moiety linked to MIT-C through a butanoic acid spacer. MC-62 exhibits significantly less biological potency as compared with the parent drug. In contrast, *N*-[4-(tetra-*O*-acetylglucopyranosyl)oxy]phenylmitomycin C (MC-77) contains the glucopyranose moiety linked to MIT-C through a phenolic spacer. This analogue generally exhibits greater antitumor activity in vitro as compared with either MC-62 or MIT-C. Thus, N_7-substituted analogues containing sugar moieties exhibit altered biological activity, the degree of which is related to the properties/structure of the spacer.

Introduction

Breast tumors respond at least initially to a wide range of systemic treatments. Following single-agent administration, response rates of 20%–38% have been reported for a variety of cytotoxic drugs, including cyclophosphamide, methotrexate, vincristine, and mitomycin C (MIT-C). MIT-C is one of the most active single agents used in breast cancer treatment, inducing objective responses in up to 38% of patients [13]. In combined modalities with other active agents, including Adriamycin, response rates as high as 73% have been reported [26]. Significant response rates have also been observed in other malignancies such as lung [2], gastric [22], colorectal [8], cervical [23, 24], and ovarian cancer [9, 29] following the administration of MIT-C.

Clinical experience with MIT-C has shown a high incidence of host toxicity [12], the most serious side effects being associated with cumulative myelosuppression. The toxicity is delayed, with the white blood cell count (WBC) nadir occurring by 3 weeks postadministration and the platelet count nadir, by 4 weeks. Although the WBC may recover rapidly, cumulative toxicity is ultimately evidenced by a progressive reduction in platelet concentration [14]. Other side effects include liver dysfunction, renal toxicity, and alopecia [39].

The antineoplastic antibiotic MIT-C is considered to be the prototype bioreductive alkylating agent [40]. Its biological activity is the result of the three functional quinone, carbamate, and aziridine groups and of their relationship to each other on the pyrolo[1,2-a]-indole nucleus. MIT-C is a poor alkylating agent in the quinone oxidation state, but reduction of this moiety results in structural transformations that can activate both the aziridine and the carbamate groups [1]. Metabolic conversion of the quinone ring to either a semiquinone or a hydroquinone species is believed to initiate drug activation and facilitate the subsequent interactions between MIT-C and DNA [10, 18]. There are two major mechanisms through which MIT-C may alter DNA structure/function. Induction of cross-linkages between DNA strands has been widely reported and reflects mono- and dialkylation mediated by the aziridine and carbamate moieties [1, 21, 38]. The most common sites for cross-linkages have been determined to lie between the two guanine residues at the N2 position [35]. Alternatively, reactive free radicals may be generated under aerobic conditions, and these can cause DNA breakage [21]. The relative importance of these two mechanisms in mediating the cytotoxic effects of MIT-C remain unclear.

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We have previously demonstrated that the addition of a sugar moiety to chlorozotocin [27] and other nitrosoureas [16, 31] results in reduced bone marrow toxicity without causing a loss of antitumor activity. The attachment of a sugar moiety to the N1 position of the parent MIT-C via a carbothioamide bridge [34] produces significantly less potent analogues that exhibit biological activity comparable with that of MIT-C against the P388 murine ascitic leukemia tumor in vivo. To increase this potency, we synthesized MIT-C sugar derivatives in which the sugar residue is attached to the N7 position via either phenol or butanoic acid spacers. A major use of these analogues would involve the treatment of breast cancer. Thus, we used five human breast-cancer cell lines growing in vitro to determine the effects of our structural modifications on the biological activity of the analogues. The MDA-MB-231 and MDA-MB-435 cell lines are representative of tumors that exhibit no detectable estrogen receptors, do not require estrogen for tumor formation in athymic nude mice, are resistant to antiestrogen therapy, and have a poor prognosis [7]. The MCF-7, ZR-75-1, and T47D cell lines retain estrogen receptor expression, absolutely require estrogen for the formation of tumors in nude mice, respond to antiestrogen therapy, and are more representative of tumors that have a better prognosis [5, 6]. The use of cell lines as an initial screen for the identification of compounds of interest is currently in line with recent changes in the drug screening program of the National Cancer Institute [3].

Materials and methods

Cell culture. All cell lines were maintained at 37° C in a humidified atmosphere comprising 5% CO_2/95% air and were routinely cultured in improved minimal essential medium (Biofluids, Rockville, Md., USA) containing phenol red and supplemented with 5% fetal calf serum (IMEM). MCF-7, T47D, and MDA-MB-231 cells were obtained from Dr. M. Lippman (Georgetown University, Washington, D. C., USA). ZR-75-1 cells were obtained from the American Type Tissue Culture (Rockville, Md., USA), and MDA-MB-435 cells were obtained from Dr. J. Price (M. D. Anderson Cancer Center, Houston, Tex., USA).

Analogues. 4-Aminophenyl-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside, *N*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-4-aminobutanamide, and mitomycin A were prepared according to established methods [11, 17, 28]. Reaction of the former with mitomycin A to produce MC-77 was performed in anhydrous methanol at room temperature under N_2. Reaction of *N*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-4-aminobutanamide with mitomycin A in anhydrous methanol at room temperature under N_2 produced MC-62.

Estimation of drug dosage. We wished to use pharmacologically relevant concentrations of MIT-C for estimations of biological activity. Since mitomycin is a cell-cycle-specific agent, exposure for a period equivalent to at least one progression of the cell cycle was required. Using a minimal plasma AUC value as described by Lankelma et al. [20], we estimated the dose range such that the concentration-time (c × t) value produced an equivalent dose following 24 h incubation in vitro. To account for potential differences between serum and tissue concentrations [36], we reduced the dose by 20 times, producing a final approximation of 1.5 nM/24 h. Consequently, cells were exposed to MIT-C and the corresponding analogues at a range of concentrations between 30 pM to 1.5 nM/24 h.

DNA synthesis. Cells (10^4) were seeded into 24-well multi-dishes and were refed 24 h thereafter with medium containing the appropriate concentrations of cytotoxic agent. The influence of MIT-C and its structural analogues on the rate of DNA synthesis was determined as described by Clarke et al. [4]. Briefly, the rate of DNA synthesis was estimated by measurement of the incorporation of [6-^{3}H]-thymidine (sp. act., 30 Ci/mmol; Amersham, Arlington Heights, Ill.) into acid-precipitable material. Cells were exposed to IMEM supplemented with 1 μCi [6-^{3}H]-thymidine/ml for 1 h and then rinsed three times in ice-cold phosphate-buffered saline. Cells were trypsinized and transferred to a Millipore filtration manifold (Millipore, Bedford, Mass., USA). Acid-insoluble material was precipitated onto GF/C filters (Whatman, Maidstone, UK) following three washes with 1.5% (v/v) trichloroacetic acid. Filters were removed and precipitated radioactivity was determined by liquid scintillation counting. Results were expressed as a percentage the value obtained for untreated cell populations.

Population growth kinetics. Cells were passaged at a split ratio of 1 : 3 at 24 h prior to seeding at approximately 1.5×10^3 cells/cm^2 into 24-well dishes (Costar, Cambridge, Mass., USA). At 24 h thereafter, cells were refed with medium containing the appropriate concentration of drug. Cells were refed with IMEM following removal of the drug 24 h later and every other day therafter. Cell numbers were determined electronically using a model Z_F Coulter Counter (Coulter Electronics, Hialeah, Fla., USA).

Anchorage-dependent and -independent colony formation. The ability of a specific drug to influence anchorage-dependent colony formation was determined as described by Van den Berg et al. [37]. Briefly, 10^4 cells were plated in 1 ml IMEM in 35-mm petri dishes (Costar), treated for 24 h with IMEM containing the appropriate concentration of drug, and refed with IMEM at the end of this period and at each subsequent 3-day interval. After 9 days, cells were stained with 1 ml staining solution (5% v/v formaldehyde, 0.1% w/v crystal violet, phosphate-buffered saline; pH 7.4).

Anchorage-independent growth was estimated by the ability of cells to form colonies during suspension in a soft agar solution [6]. Bottom layers of agar were prepared by allowing 1 ml IMEM containing 0.6% (w/v) agar (Difco, Detroit, Mich., USA) to solidify in 35-mm petri dishes. Cells were passaged at a split ratio of 1 : 3 at 24 h prior to seeding at approximately 1.5×10^3 cells/cm^2 into 25-cm^2 flasks. After 24 h incubation, cells were refed with medium containing cytotoxic drug for a further 24 h. Subsequently, cells (5×10^3 MDA-MB-231 and 10^4 MCF-7) were suspended in 1 ml IMEM containing 0.6% (w/v) agar at 41° C. This agar solution was placed over the solid bottom agar layer, allowed to solidify, and incubated for 10 days at 37° C in a humidified atmosphere comprising 5% CO_2 : 95% air. Colonies measuring 60 μm in diameter were counted using an Omnicon 3600 electronic image analyzer (Artek Systems Corporation, Farmingdale, N. Y., USA). Results were expressed as a percentage of the value obtained for untreated cell populations.

Results

Inhibition of DNA synthesis

The cytotoxic effects of MIT-C are thought to reflect largely the result of its interactions with DNA. Consequently, we initially determined the ability of MIT-C and its analogues to inhibit the rate of incorporation of [^{3}H]-deoxythymidine (TdR) into DNA. Pharmacologically relevant concentrations of MIT-C and equivalent concentrations of MC-77 induced a significant and dose-dependent inhibition of DNA synthesis in all five cell lines. At equimolar concentrations, we consistently observed a significantly greater inhibition of DNA synthesis following treatment with MC-77 as compared with the parent compound in MCF-7 (Fig. 1a), MDA-MB-231 (Fig. 1b), and T47D cells (Fig. 2b). For example, in MDA-MB-231 cells,

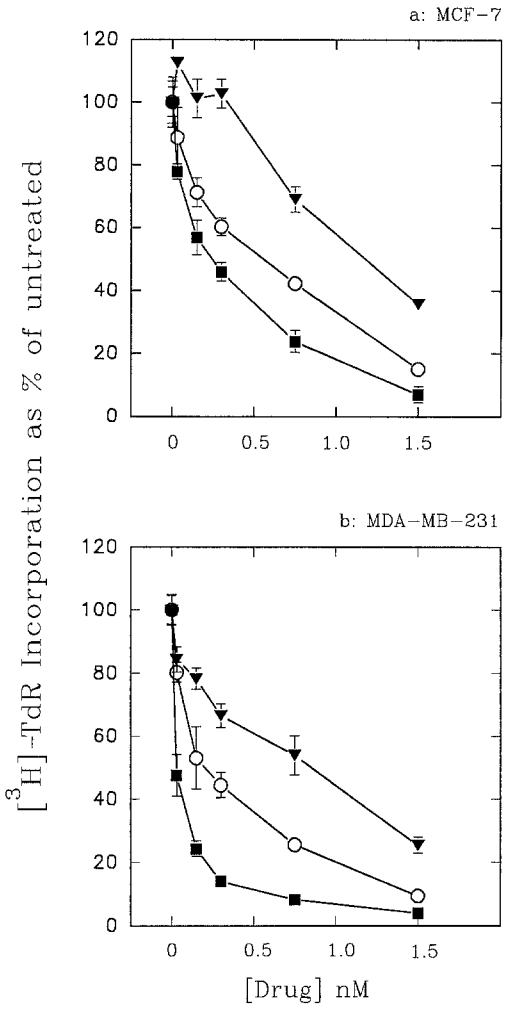

Fig. 1 a, b. The effect of MIT-C (○), MC-77 (■), and MC-62 (▲) on [6-^{3}H]-thymidine incorporation into acid-precipitable material in **a** MCF-7 and **b** MDA-MB-231 cells. Cells were exposed to increasing concentrations of drug for 24 h prior to determinations of nucleotide incorporation. Data represent mean values ± SD for 4 determinations. The absence of error bars indicates that the SD was smaller than the symbol representing the mean value

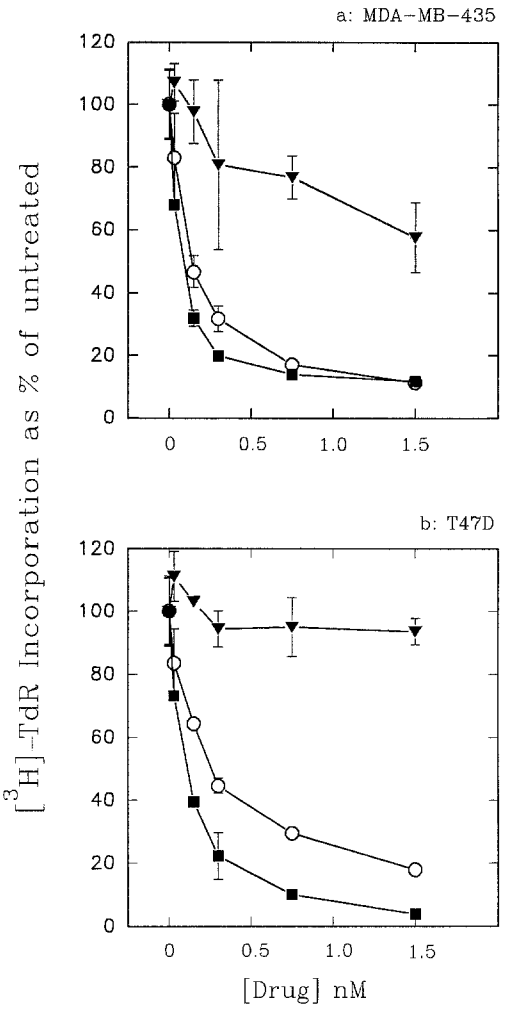

Fig. 2 a, b. The effect of MIT-C (○), MC-77 (■), and MC-62 (▲) on [6-^{3}H]-thymidine incorporation into acid-precipitable material in **a** MDA-MB-435 and **b** T47D cells. Cells were exposed to increasing concentrations of drug for 24 h prior to determinations of nucleotide incorporation. Data represent mean values ± SD for 4 determinations. The absence of error bars indicates that the SD was smaller than the symbol representing the mean value

0.15 nM MC-77 inhibited nucleotide incorporation by approximately 80%, whereas MIT-C inhibited incorporation by only 47% (P <0.002; Student's t-test). A 5-fold increase in the concentration of the parent drug was required to produce an effect equivalent to that induced by 0.15 nM MC-77 in these cells. The cytotoxicity of MC-77 was approximately 2-fold that of MIT-C at a concentration of 0.75 nM in MCF-7 cells (P <0.001; Student's t-test). In T47D cells, 0.3 nM MC-77 inhibited nucleotide incorporation by 77.8%, whereas MIT-C inhibited incorporation by only 55.5% (P <0.002; Student's t-test). A 2- to 6-fold increase in the concentration of MIT-C was required to inhibit nucleotide incorporation by approximately 80% (Fig. 2b). The inhibitory effects of MIT-C and MC-77 were equivalent in MDA-MB-435 (Fig. 2a) and ZR-75-1 cells (Fig. 3). In marked contrast, MC-62 was significantly less potent than both MIT-C and MC-77 in all five cell lines tested.

Inhibition of the rate of cell proliferation

Figure 4 shows the effect of MIT-C, MC-77, and MC-62 on the population kinetics of MDA-MB-231 cells. MC-62 failed to influence the rate of MDA-MB-231 growth markedly. Both MIT-C and MC-77 significantly inhibited the rate of cell proliferation. However, there was no clear dose-response relationship, and only treatment with 1.5 nM MC-77 reduced the number of cells to a level significantly below the baseline value. In MCF-7 cells (Fig. 5), we also failed to observe any significant dose-response relationship. None of the drug concentrations examined were capable of inhibiting cell number the number of cells to a level below the baseline value in these cells.

Colony-forming ability

MC-77 and the parent compound induced a significant reduction in anchorage-dependent colony-forming ability

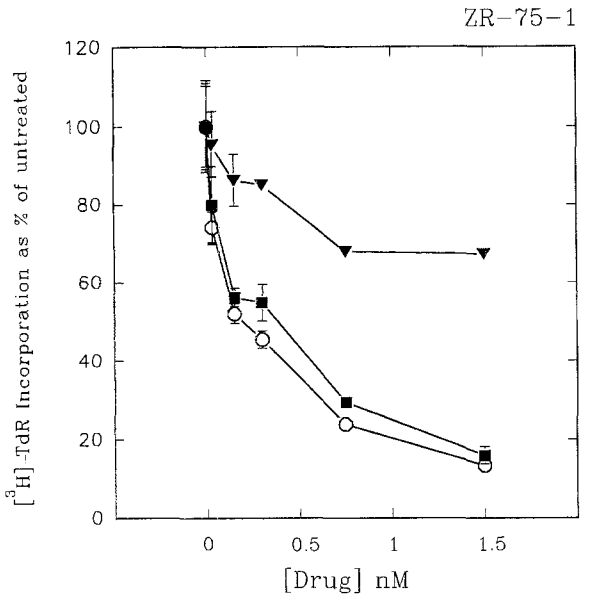

Fig. 3. The effect of MIT-C (○), MC-77 (■), and MC-62 (▲) on [6-^{3}H]-thymidine incorporation into acid-precipitable material in ZR-75-1 cells. Cells were exposed to increasing concentrations of drug for 24 h prior to determinations of nucleotide incorporation. Data represent mean values ± SD for 4 determinations. The absence of error bars indicates that the SD was smaller than the symbol representing the mean value

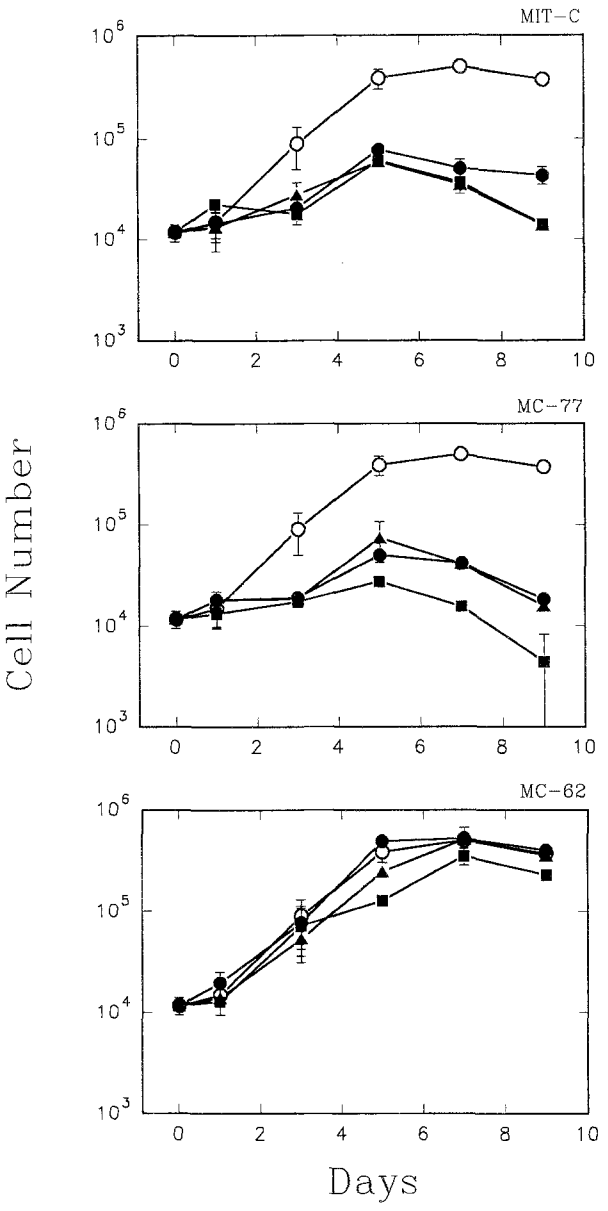

Fig. 4. The inhibitory effects of MIT-C, MC-62, and MC-77 on the population growth kinetics of MDA-MB-231 cells. Cells were exposed to the appropriate concentration of drug for 24 h. ○, Untreated; ▲, 0.25 nM; ●, 0.75 nM; ■, 1.5 nM. Data represent mean values ± SD for 3 determinations. The absence of error bars indicates that the SD was smaller than the symbol representing the mean value

at a concentration of 0.25 nM in both MCF-7 and MDA-MB-231 cells (Fig. 6). We estimate the inhibition induced by 0.25 nM MC-77 to be approximately 5- to 10-fold that induced by an equimolar concentration of MIT-C. Treatment with MC-62 at concentrations of up to 0.25 nM did not significantly influence anchorage-dependent colony formation in either cell line (data not shown).

The anchorage-independent assay determines the ability of cells to form colonies during growth in suspension in a soft agar solution. In MDA-MB-231 (Fig. 6a) and MCF-7 cells (Fig. 6b), both MIT-C and MC-77 induced dose-dependent reductions in cell survival. However, MC-77 appeared to be up to 10 times more cytotoxic than the parent drug. In agreement with the other endpoints, MC-62 consistently failed to exhibit substantial biological activity in either MCF-7 or MDA-MB-231 cells.

Discussion

Effects of structural modification at the C_7 position of MIT-C on biological activity

Structure-activity studies using chlorozotocin [27] and other sugar-containing nitrosoureas [16, 31] have demonstrated that the addition of a sugar moiety results in reduced bone marrow toxicity without causing a loss of antitumor activity. Our laboratory has shown that the attachment of a cytotoxic nitrogen mustard to the C6 position of galactose produces full antitumor activity along with a decrease in hematotoxicity [32]. Reduced myelotoxicity correlates with a selective reduction in the alkylation of transcriptionally active chromatin in bone marrow as compared with tumor cells [15, 27]. To investigate the potential for lower bone marrow toxicity in cytotoxic compounds containing sugar residues and to increase the water solubility of these agents, we have previously synthesized and tested several N1-sugar-containing MIT-C analogues. In these compounds a sugar residue was attached to the N1 position, of the parent MIT-C via a carbothioamide bridge [34]. Although they are significantly less potent than MIT-C, these analogues exhibit biological activity comparable with that of the parent compound MIT-C against the P388 murine ascitic leukemia tumor in vivo. In contrast to the highly myelosuppressive parent drug, optimal antitumor activity is achieved at doses of the analogues that induce only limited leukopenia [34].

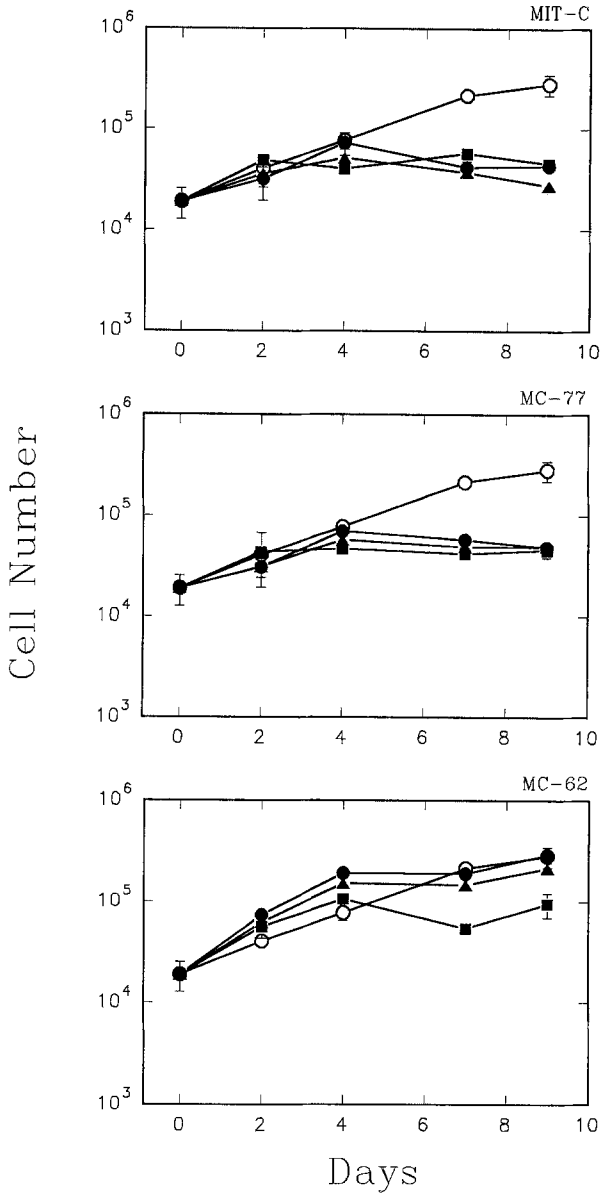

Fig. 5. The inhibitory effects of MIT-C, MC-62, and MC-77 on the population growth kinetics of MCF-7 cells. Cells were exposed to the appropriate concentration of drug for 24 h. ○, Untreated; ▲, 0.25 nM; ●, 0.75 nM; ■, 1.5 nM. Data represent mean values ± SD for 3 determinations. The absence of error bars indicates that the SD was smaller than the symbol representing the mean value

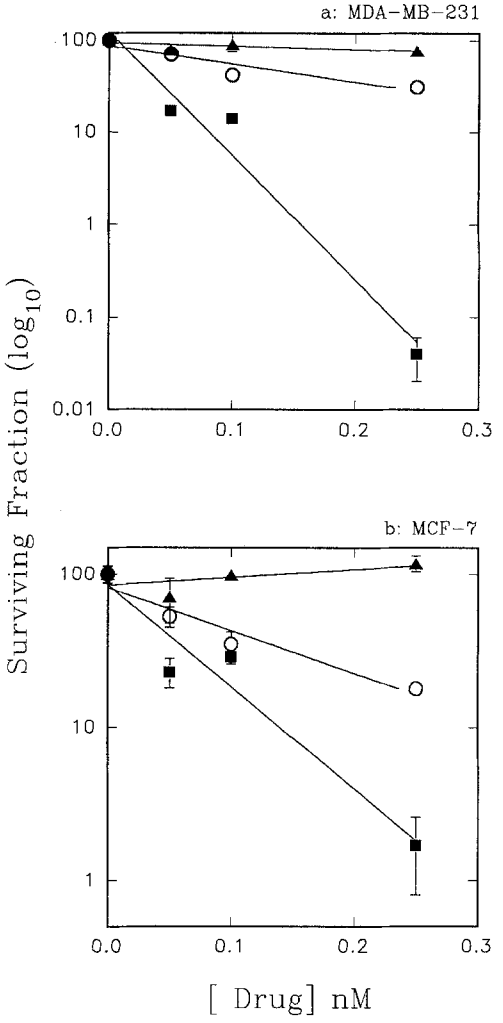

Fig. 6a, b. The effects of MIT-C (○), MC-62 (▲), and MC-77 (■) on anchorage-independent colony formation in **a** MDA-MB-231 and **b** MCF-7 cells. Cells were exposed to increasing concentrations of drug for 24 h prior to seeding into IMEM containing soft agar. Data represent mean values ± SD for 3 determinations and are expressed as a percentage of the values obtained for untreated cell populations. The *lines* represent the results of least-squares linear regression analysis. The absence of error bars indicates that the SD was smaller than the symbol representing the mean value

In the following discussion, we report on our initial investigations of the effect of N7-substituted spacer molecules on the biological activity of two MIT-C/sugar analogues. The data from all four biological endpoints clearly indicate that MC-77 is more bioactive than either MIT-C or MC-62 in both MCF-7 and MDA-MB-231 cells. In all five cell lines, MC-62 is significantly less active than both MIT-C and MC-77. Studies on the structure-activity relationships of N7-substituted MIT-C analogues have shown that groups that facilitate the bioactivation of the quinone in MIT-C generally result in more biologically active analogues. The 7-ethylamino derivatives frequently exhibit weak antitumor activity and are relatively resistant to reduction [19]. The 7-anilino analogues are more easily reduced and clearly express increased antitumor activity as compared with the parent compound [30]. MC-62 is essentially a sugar propylamino derivative and MC-77 is a sugar anilino congener. Since the only structural difference between MC-62 and MC-77 is the spacer (butanamide vs phenol), we think that the resultant differences in the reduction potential of the two compounds may be responsible for the observed differences in their in vitro biological activity.

There are also significant differences among these cell lines with respect to their relative sensitivities to each of the three drugs. For example, MC-77 is more active than MIT-C, but only in MCF-7, MDA-MB-231, and T47D cells. The ability of MC-62 to inhibit the rate of DNA synthesis is apparently greater in MCF-7 and MDA-MB-231 cells as compared with T47D, ZR-75-1, and MDA-MB-435 cells. However, the inhibitory effects of MIT-C

are relatively consistent among the cells lines, e.g., 0.25 nM MIT-C/24 h inhibits DNA synthesis by approximately 35%–40%. The MDA-MB-435 cells appear to be slightly more sensitive to MIT-C than are the other cell lines. These data suggest that in addition to any structural constraints on cytotoxicity, there are either intracellular pharmacologic or metabolic differences between cells that also contribute to the degree of cytotoxicity. Significantly these differences do not appear to be related to the estrogen receptor (ER) status of the cells or to their degree of hormone dependence. It is also unlikely that this differential sensitivity reflects kinetic differences in the cell populations. In general, the ER-positive MCF-7, T47D, and ZR-75-1 cells exhibit approximately equivalent population-doubling times during in vivo growth. The ER-negative MDA-MB-231 and MDA-MB-435 cells also exhibit equivalent population-doubling times, although these populations tend to cycle more rapidly than do the ER-positive cells.

Choice of endpoint and estimation of cytotoxicity

The ability of a cytotoxic agent to eradicate a tumor requires that the cells be rendered at least reproductively inert. This is sufficient to prohibit the expansion of the primary tumor and the successful establishment of proliferating metastases elsewhere. The cytotoxic effects of some antineoplastic drugs are not fully evidenced until the target cell and its progeny have completed one or more cell divisions. Since the critical endpoint is the extent of reproductive cell death [37], endpoints that rely solely on post treatment cell numbers may underestimate the cell kill. Cells that survive but are either reproductively dead or capable of only a limited number of cell divisions can increase the apparent survival fraction. For our analysis, we chose to implement and compare endpoints based on cell population kinetics, colony formation, and nucleotide incorporation.

Interpretation of the effects of cytotoxic drugs on the rate of cell proliferation is frequently difficult. Cell killing is clearly evidenced only when the number of cells has fallen significantly below the baseline value [37]. On the basis of our cell population-growth data alone, we can report cell death only at high doses of MC-77. However, both colony-formation assays clearly indicate the presence of drug-induced reproductive cell death.

The most acurate methods for determining cell kill measure the ability of a single cell to produce a clone of ≥ 50 cells; this requires that the parental cell and its daughters complete the equivalent of ≥ 6 divisions [6]. Anchorage-dependent colony formation is regarded as being resistant to perturbations in either cell population-growth kinetics of tritiated nucleotide incorporation. However, some cell lines fail to form appropriate, tightly packed colonies when they are grown on a solid plastic surface, particularly MDA-MB-231 cells. This partly reflects the mobility of epithelial cells growing on a solid substratum [33]. In contrast, colonies formed in a soft agar medium are clearly delineated and easily identified and enumerated electronically.

We have previously observed that some drug concentrations may produce an apparent increase in precursor incorporation [4, 37], presumably due to the induction of a nonlethal block in the S phase. Although they are incapable of reproducing, intact isolated nuclei can also incorporate nucleotides into nucleic acids. This process is often used as a measure of the functional capabilities of isolated nuclei [25]. Nevertheless, we consistently observed a clear correlation between [^{3}H]-TdR incorporation into acid-precipitable material and both anchorage-dependent and anchorage-independent colony formation.

In conclusion, in vitro analyses of MIT-C analogues indicate that tritiated precursor incorporation into acid-precipitable material can provide a useful and rapid initial screen for biological activity. Substitution at the N7 position can produce analogues exhibiting altered potency. However, the degree of biological activity is clearly related to the attached moieties and to the effects of these spacers on reduction potential. Since we also anticipate a reduction in toxicity as a result of increased water solubility, we are currently evaluating these compounds in vivo for both their host toxicity profiles and their antitumor activities.

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