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The Pyrimido-pyrimidine Derivatives RA 233 and RX-RA 85 affect Growth and Cytoskeletal Organization of Rat Mammary Adenocarcinoma Cells*

ROSEMARIE B. LICHTNER and GARTH L. NICOLSON†

Department of Tumor Biology, The University of Texas M.D. Anderson Hospital and Tumor Institute, Houston, Texas 77030 U.S.A.

Abstract—*The pyrimido-pyrimidine derivatives RA 233 and RX-RA 85, which are potent inhibitors of platelet and tumor phosphodiesterase, were developed as antitumor agents. When tested by us, these drugs were cytostatic at low concentrations and produced dramatic changes in cell shape and organization of cytoskeletal structures in cultured MTF7 cells derived from the rat 13762NF mammary adenocarcinoma. At high concentrations (up to 600 μg/ml) RA 233 was cytostatic but not cytotoxic to MTF7 cells during a 24 hr incubation in vitro, whereas RX-RA 85 was cytotoxic at concentrations above 4 μg/ml. These drugs caused MTF7 cells to elongate and form numerous vacuoles, which surrounded the cell nucleus. Treatment of MTF7 cells with RA 233 or RX-RA 85 enhanced microtubular organization concomitant with a decrease in microfilament organization. In contrast, treatment of MTF7 cells with 1 mM dibutyryl cAMP resulted in an enhanced organization of microtubules but had no effect on microfilament organization. Previous studies suggested that RA 233 and RX-RA 85 increase cAMP levels in 2 other cell clones of rat 13762NF mammary adenocarcinoma by inhibiting phosphodiesterases. However, additional sites of drug action should also be considered based on the effects of these drugs on microfilament systems and cell vacuoles.*

INTRODUCTION

THE cytoskeleton of normal and tumor cells plays a major, though not entirely elucidated, role in the maintenance and modulation of cell shape and secretory processes, distribution of cell organelles such as chromosomes during mitosis and modulation of cell surface domains and cell motility. The major cytoskeleton components of mammalian cells are microfilaments (MF), microtubules (MT) and intermediate filaments (IF), and these elements are characterized by specific biochemical and immunological properties [1]. Transformed cells generally exhibit a reduced organization of MF and occasionally MT [2, 3], although the usual organization can reappear with reversion to a more normal phenotype [4].

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†To whom correspondence and requests for reprints should be addressed.

There is little information concerning the role of MF and MT in specific steps of tumor metastasis. The involvement of the cytoskeleton in blood-borne lung colonization of B16 melanoma cells was shown by incubating B16 cells with cytoskeleton-disrupting agents, such as cytochalasin B [5] and tertiary amine local anesthetics [6]. These agents caused cell rounding, loss of cytoskeletal organization, reduced adhesion to endothelial cells, and lowered ability to form experimental lung metastases. Mareel and De Mets [7] demonstrated that invasion of tumor cells into chick heart cell aggregates was blocked by MT inhibitors.

Cytoplasmic MT and MF are regulated by intracellular Ca^{2+} and cAMP concentrations [8], and the cAMP concentrations depend, in turn, on the activities of adenylate cyclases and phosphodiesterases. The pyrimido-pyrimidine derivatives RA 233 and RX-RA 85 are described by the manufacturer as potent platelet (H. Weisenberger, unpublished results) and tumor cell (R. Zimmermann, unpublished results) phosphodiesterase (PDE) inhibitors.

RA 233 has been investigated for its antimetastatic activity in a clinical trial [9], and both drugs have been tested for inhibition of metastasis in a number of animal tumor systems [10, 11]. There is some circumstantial evidence that indicates interference of RA 233 with the functions of cytoskeletal elements. RA 233 has been shown to have dramatic effects on the cell cycle distribution of 3LL and B16 tumor cells grown *in vitro*, and it results in accumulation of cells in G_2 + M with concomitant increases in cell volume and the number of tetraploid cells [12]. These results suggest that mitotic inhibition might be caused by drug interference with the reorganization of cytoskeletal structures important for mitosis. These effects were not due to any cytotoxic action of the drugs [12–15].

We investigated the effects of the pyrimido-pyrimidine derivatives RA 233 and RX-RA 85 on the cell growth properties and MT and MF cytoskeletal organization in a cloned rat mammary adenocarcinoma cell line.

MATERIALS AND METHODS

Cell lines and culture conditions

The tumor cell clone used in these studies is from the 13762NF mammary adenocarcinoma syngeneic to F344 rats [16]. The biological properties of several spontaneously metastasizing clones and cell sublines derived from this tumor are described elsewhere [17]. Clone MTF7 (T11) cl.2 of intermediate metastatic potential was grown in a 37°C humidified atmosphere (95% air, 5% CO_2) in alpha-modified minimum essential medium (AMEM) supplemented with 5% fetal bovine serum (Flow Laboratories) without antibiotics. Cells were grown to 60–80% confluency, harvested using 0.25% trypsin-2 mM EDTA in Dulbecco's phosphate-buffered saline (DPBS) free of calcium and magnesium and diluted (1 : 50) into fresh medium with serum. The MTF7 cells were used at cell passages less than 15.

Drugs

The pyrimido-pyrimidine derivatives RA 233 and RX-RA 85 [18] (from Dr. Karl Thomae GmbH, Biberach, West Germany) have been classified by the manufacturer as very potent inhibitors of platelet and tumor cell phosphodiesterases (PDE) (H. Weisenberger, R. Zimmermann, unpublished results), with RX-RA 85 ($EC_{50} \sim 2 \times 10^{-9}$M) being approx. 10^4 times more potent than RA 233 ($EC_{50} \sim 2 \times 10^{-5}$M) in inhibiting platelet PDE (H. Weisenberger, unpublished results). The drugs were solubilized in hydrochloric acid and diluted in PBS as described previously [10, 11]. RA 233 is stable in solution for at least 1 week, but RX-RA 85 solutions were prepared daily. N^6, O^2-dibutyryl adenosine 3′ : 5′-cyclic monophosphate (Bt_2cAMP) was purchased from Sigma; its purity was > 96%.

In vitro *cell growth*

For cell growth studies, 10^5 cells taken from logarithmically-growing cultures were plated onto Corning 60 mm dia. tissue culture dishes containing 3 ml medium. Drug or control solutions were added after 24 hr without changing the medium, and cell numbers were determined after an additional 24 hr when the cells were in exponential growth phase by detaching the cells with trypsin/EDTA and counting them using a Coulter electronic counter (Coulter Electronics, Inc., Hialeah, FL).

Optical microscopy

Untreated and drug-treated tumor cells were examined by phase microscopy using a Zeiss photomicroscope equipped with Kodak Panax 32 film. Cells grown on plastic dishes were gently washed once with DPBS at 37° C and then fixed at this temperature with 1% glutaraldehyde, 0.125 M sodium cacodylate buffer, pH 7.3, for 30 min.

Fluorescent labeling of cells

Cells (10^5) from exponentially-growing cultures were seeded onto a glass coverslip (22 mm dia.) and after 24 hr incubation the drugs added and the incubation continued. Preparation of Triton X-100-resistant cytoskeletons was performed essentially as described by Brinkley *et al.* [19]. Extracted cytoskeletons were treated with sheep antitubulin antibody (Southern Biotech. Ass. Inc., Birmingham, AL) and then with the second antibody (rabbit anti-sheep IgG) labeled with fluorescein (Miles Scientific, Naperville, IL) to localize MT. The actin-containing

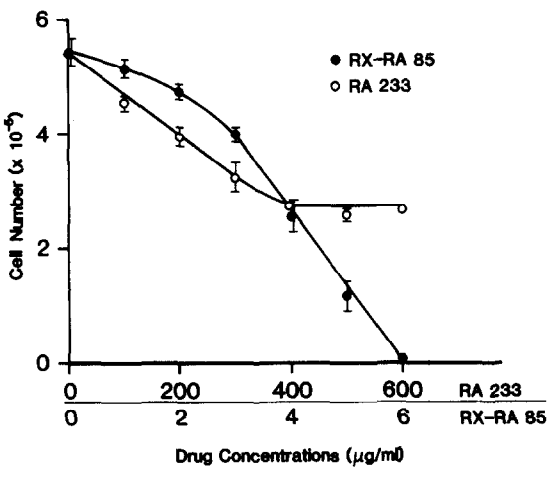


Fig. 1. MTF7 cells were incubated and cell growth determined in the presence of various RA 233 (○) *concentrations and RX-RA* 85 (●) *concentrations. Cells* (10^5) *were plated on day* 0, *after* 24 hr *the drugs added, and after* 24 hr *cell number estimated with cultures still in exponential growth phase. Each experiment was performed* 3 *times with triplicate samples (mean ± S.E.M.).*

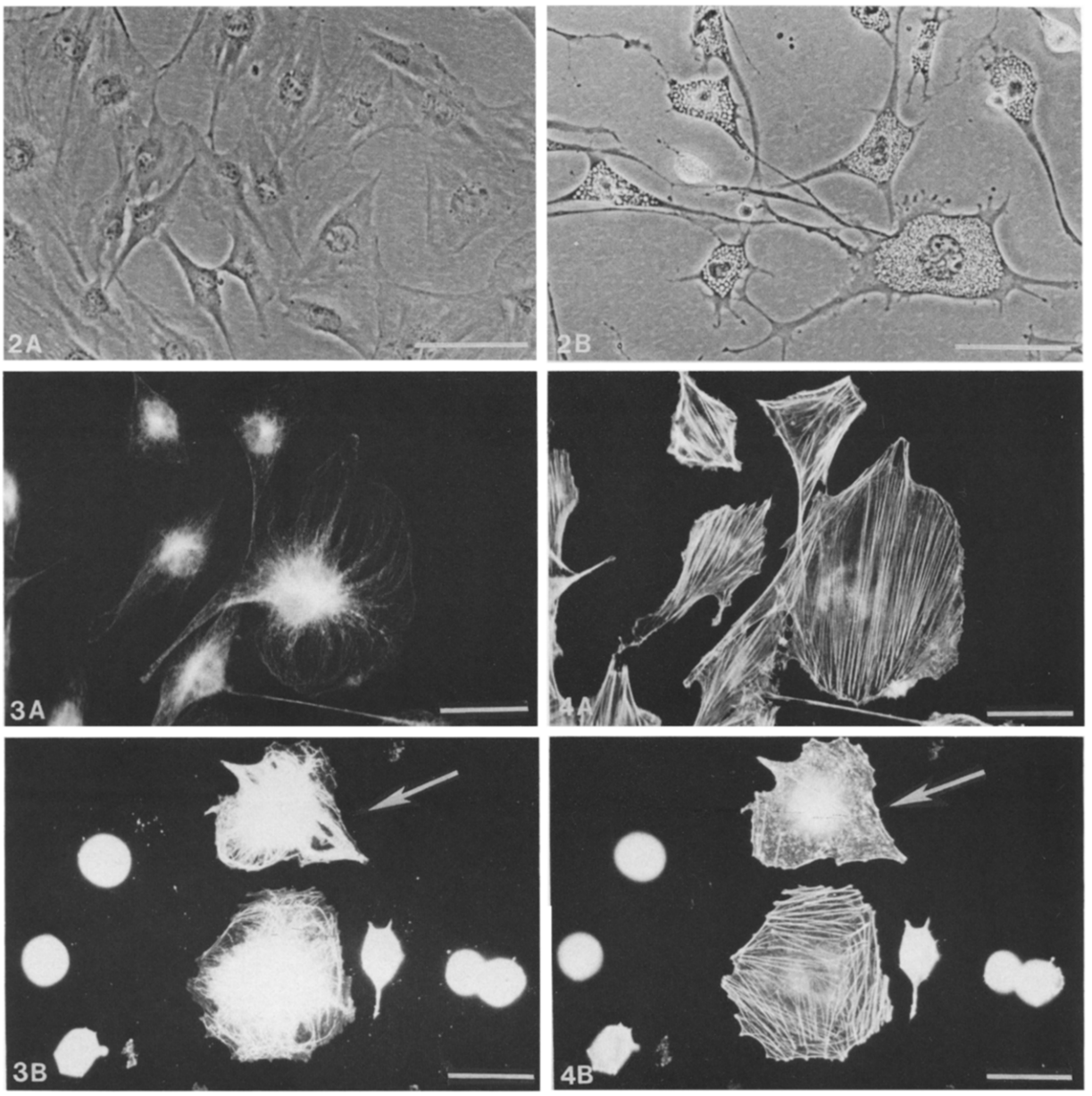
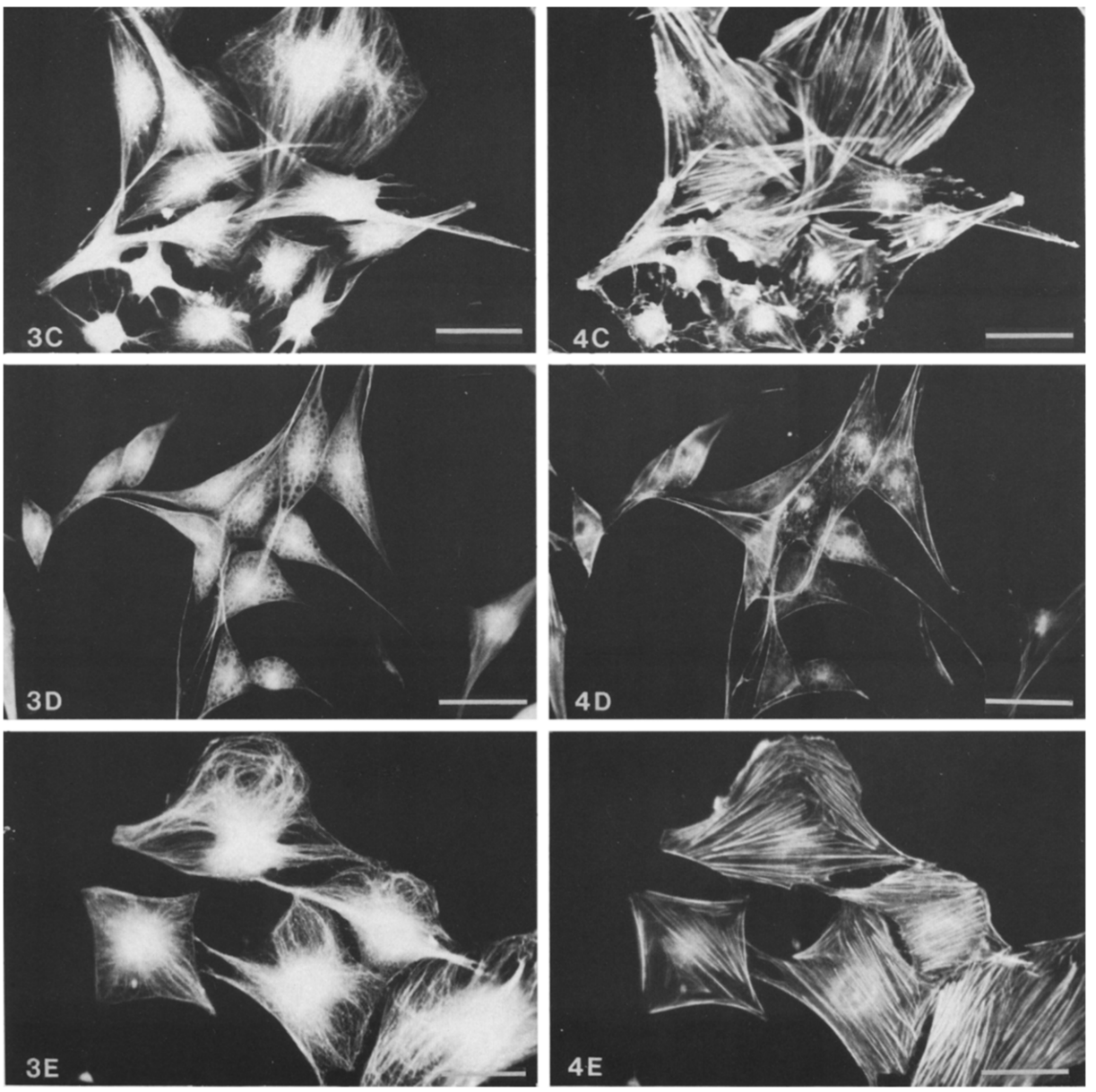


Fig. 2. *Phase contrast micrograph of untreated MTF7 cells*; (*A*) *control, untreated cells*; (*B*) *cells treated for* 24 hr *with* 400 μg/ml *RA* 233 (*bar* = 30 μm).

Fig. 3. *Immunofluorescence distribution of MT in MTF7 cells.* (*A*) *control, untreated cells*; (*B*) *cell treated for* 1 hr *with* 4 μg/ml *RX-RA* 85; (*C*) *cells treated for* 24 hr *with* 100 μg/ml *RA* 233; (*D*) *cells treated for* 24 hr *with* 400 μg/ml *RA* 233; (*E*) *cells treated for* 7 hr *with* 1 mM Bt_2cAMP (*bar* = 30 μm).

Fig. 4. *Fluorescent phalloidin distribution of MF in MTF7 cells.* (*A*) *control, untreated cells*; (*B*) *cells treated for* 1 hr *with* 4 μg/ml *RX-RA* 85; (*C*) *cells treated for* 24 hr *with* 100 μg/ml *RA* 233; (*D*) *cells treated for* 24 hr *with* 400 μg/ml *RA* 233; (*E*) *cells treated for* 7 hr *with* 1 *mM* Bt_2cAMP (*bar* = 30 μm).


3C
4C
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MF in the same cytoskeleton preparation were subsequently stained with rhodamine-labeled phalloidin (Molecular Probes, Inc., Junction City, OR). Microscopic observations were performed using a Leitz photomicroscope equipped for fluorescein and rhodamine fluorescence. Kodak Tri-X film was used for photography. Within 1 set of experiments the same exposure and film development times were used for tubulin and actin staining.

RESULTS

Incubation of MTF7 tumor cells for 24 hr with RA 233 or RX-RA 85 reduced cell numbers compared with controls ($EC_{50} \sim 300$ μg/ml or 3 μg/ml, respectively) (Fig. 1). RA 233 used at concentrations above 400 μg/ml was mainly cytostatic rather than cytotoxic, and increasing drug concentrations up to 600 μg/ml did not result in increased cytotoxicity. In contrast, concentrations of RX-RA 85 above 3 μg/ml resulted in cell death, which microscopic observations of untreated and drug-treated MTF7 cells in tissue culture confirmed.

RA 233 or RX-RA 85 treatment of MTF7 cells resulted in changes in cell morphology. MTF7 control cells spread with epithelial morphology and were very heterogeneous in cell shape and size (Fig. 2A). Incubating MTF7 cells with either drug caused slight retraction of the cells and led to the formation of very small vacuoles around the nuclei. These changes were dose-dependent and became more obvious with time. After a 24-hr exposure to 400 μg/ml RA 233 (Fig. 2B) or 3 μg/ml RX-RA 85 (data not shown) nearly complete retraction of the cytoplasm occurred, leading to star-shaped cells with long cellular processes. Occasionally these cells were completely filled with small vacuoles. In addition, some round, mitotic-like cells were seen after treatment of MTF7 cells with high concentrations of RA 233. RA 233 concentrations up to 600 μg/ml and RX-RA 85 concentrations up to 3 μg/ml caused cell shape changes and formation of cytoplasmic vacuoles that were completely reversible: upon removal of drugs, washing with DPBS, and addition of fresh growth medium, the MTF7 cells reverted to normal morphologies within 6 hr and then began proliferating.

The dramatic cell shape changes after treatment with RA 233 or RX-RA 85 indicated that these drugs induce changes in the organization of cytoskeletal structures; we therefore examined the organization of MF and MT in untreated and drug-treated MTF7 cells by immunofluorescence. Figures 3A and 4A demonstrate the organization of MT and MF in control MTF7 cells, respectively. In these figures, the MT originate from 1 bright spot near the nucleus, termed the MT-organizing center, and extend to the cell periphery, where they often parallel the cell border (Fig. 3A). MTF7 cells showed well-developed MF-containing stress fibers that span the entire cell (Fig. 4A). Incubating MTF7 cells with RX-RA 85 or RA 233 resulted generally in the formation of more enhanced MT complexes as indicated by stronger MT fluorescence and the formation of thicker MT bundles (Figs. 3B and 3C). However, the organization of MF decreased with drug treatment (Figs. 4B and 4C). Although treatment of cells with lower drug concentrations (1 μg/ml RX-RA 85 or 100 μg/ml RA 233) for 1 hr did not result in any obvious changes in cytoskeletal organization (data not shown), increasing drug concentrations or incubation times caused dramatic changes in MT and MF organization. Incubating MTF7 cells for 1 hr with 4 μg/ml RX-RA 85 resulted in a stronger MT complex formation (Fig. 3B). As the figure shows, several cells are rounded up and no structured MT are visible. The organization of MF into stress fibers decreased concomitantly with the increased formation of MT complex (Fig. 4B). The cell with the most visible disarray of MT (arrow) exhibited only a very few stress fibers. In Fig. 3C (100 μg/ml RA 233 for 24 hr) a cluster of small cells shows intense fluorescence staining for tubulin while the length and numbers of MF in these cells are decreased (Fig. 4C). In this figure, 2 cells have just completed mitosis and the midbody is clearly visible. Incubating MTF7 cells for 24 hr with 400 μg/ml of RA 233 resulted in retracted or elongated cells that stained intensely for MT (Fig. 3D). Since cell shape changed so dramatically, the intense fluorescence staining for tubulin cannot be taken as an indication of increased MT. Although the formation of stress fibers was decreased, MTF7 cells still exhibited intense staining for F-actin (Fig. 4D). In Fig. 4D, numerous vacuoles can also be identified near the MT complex, and these are apparently the same type of vacuoles shown in Fig. 2B.

In addition, we tested the effects of Bt_2cAMP on the cytoskeletal organization in MTF7 cells, since RA 233 and RX-RA 85 have been described as potent inhibitors of tumor cell PDE (R. Zimmermann, unpublished results). Incubating cells with 1 mM Bt_2cAMP for 3–24 hr resulted in increased MT organization, like that found with RA 233 or RX-RA 85 (Fig. 3E). Although Bt_2cAMP resulted in the formation of more pronounced MT complexes and thicker MT bundles, it did not influence MF organization in these cells (Fig. 4E). In addition, dramatic shape changes and vacuole formation were not apparent in Bt_2cAMP-treated (up to 10 mM) MTF7 cells (data not shown).

DISCUSSION

The cell cytoskeleton is thought to function as a linkage between the nucleus and the plasma membrane [2, 3], and it apparently plays a regulatory role in initiating DNA synthesis [20] and regu-

lating the mobility of surface receptors [2, 21] and enzymes [22]. Thus, cytoskeleton alterations could result in changes in cell behavior.

The effects of cAMP on cytoskeletal organization and the resulting alterations of cell functions have been investigated in established cell lines in tissue culture (for reviews see [8], [23]) and normal cells, such as primary cultures and lymphocytes (for review see [23]). For example, incubation of rat primary neonatal hepatocytes [24] or primary heart cell cultures [25] with cAMP or Bt_2cAMP resulted in changes in cell shape, ultrastructure and MT organization. In established cell lines, treatment with Bt_2cAMP also resulted in shape changes. In several cell lines MT organization was increased [26, 27] by such treatment, whereas in other cell types dramatic alterations in the MT network were not found [4]. There were also disparate effects of Bt_2cAMP on MF organization in different cells. In the study of Bloom and Lockwood [4], Bt_2cAMP reversibly induced the formation of MF stress fibers; however, Willingham and Pastan [27] were undecided on whether the number of MFs changed or whether there was redistribution of existing MF structures. Willingham and Pastan [27] have proposed a model for the role of cAMP in the control of cell shape and motility. In this model high cAMP levels are thought to promote assembly of MT and inhibit MF-dependent functions.

A functional role for cAMP in the regulation of MT assembly was proposed based on the observation that cAMP causes pronounced tubulin-related morphological changes in cells. In addition, cAMP-dependent protein kinase may be intimately associated with purified MT, since a MT component (MAP_2) is preferentially phosphorylated in a cAMP-dependent manner *in vitro* and *in vivo* [8]. Incubating MTF7 cells with 1 mM Bt_2cAMP resulted in an increased organization of MT with no apparent effects on MF organization or cell shape. RA 233 and RX-RA 85 have been classified by the manufacturer as potent inhibitors or tumor cell PDE (R. Zimmermann, unpublished results). We have shown recently that RA 233 inhibits to similar degrees cAMP-dependent PDE in cell lysates and cAMP metabolism in intact 13762NF rat mammary adenocarcinoma cells [28]. Thus, the changes described in MT organization in MTF7 cells treated with RA 233 and RX-RA 85 mimicked the changes observed with Bt_2cAMP and might be caused by altered cAMP levels. We also conclude that additional sites of action at which these drugs might affect the changes in MF organization and formation of vacuoles should be considered. Using Sertoli cells it was reported that the gross cell shape is not primarily controlled by the integrity of the cytoplasmic MT network but by the MF network [8], which might also apply for MTF7 cells. We feel that RA 233 and RX-RA 85 do not act directly on F-actin since we have not seen aggregates of F-actin similar to those found after microinjection of phalloidin into cells [29]. Thus RA 233 and RX-RA 85 probably act indirectly via MF regulatory mechanisms.

Dipyridamole, another pyrimido-pyrimidine derivative has also been shown to induce cell shape changes [30] and reduce MF organization in cultured mouse embryo cells [31]. However, the effects of dipyridamole on MT organization were not investigated. Dipyridamole is a weak PDE inhibitor (measured with platelet PDE) (Weisenberger, unpublished results) and a very potent inhibitor of nucleoside transport [32]. Therefore, the pyrimido-pyrimidine derivatives may generally affect cytoskeletal organization in cells. These alterations in cytoskeletal organization may, in turn, induce a number of changes in tumor cell behavior that are important in tumor cell growth, invasion and metastasis.

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