

N-Substituted 4-Aminobenzamides (Procainamide Analogs): An Assessment of Multiple Cellular Effects Concerning Ion Trapping^[S]

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ABSTRACT

Procainamide and related triethylamine-substituted 4-amino-benzamides, such as metoclopramide and declopramide, exert cellular effects potentially exploitable in oncology at millimolar concentrations (DNA demethylation, nuclear factor- κ B inhibition, apoptosis) and display anti-inflammatory properties. However, these drugs induce massive cell vacuolization at similar concentrations, a response initiated by vacuolar (V-) ATPase-dependent ion trapping into and osmotic swelling of acidic organelles. We have examined whether this overlooked response might be related to the effects on cell proliferation and viability using cultured vascular smooth muscle cells and tumor-derived cell lines (Morris 7777 hepatoma, HT-1080 fibrosarcoma). Giant vacuole formation, of confirmed *trans*-Golgi origin (labeled with C5-ceramide, p230, golgin-97), is a cellular response to all tested amines in the series (≥ 2.5 mM), including triethylamine. These drugs and the V-ATPase inhibitor bafilomycin A1 inhibited smooth muscle cell proliferation, suggesting

that acidification of a cellular compartment is essential to cell division. The cytotoxicity was maximal with metoclopramide, and this effect was minimally influenced by bafilomycin A1; furthermore, metoclopramide (2.5 mM) induced apoptosis in tumor cells as judged by poly(ADP-ribose)polymerase (PARP) cleavage. Triethylamine and procainamide exhibit a low level of cytotoxicity variably reduced by bafilomycin cotreatment. In Morris cells, the secretion of α -fetoprotein is inhibited by amines, consistent with the impairment of the secretory pathway. The most highly substituted 4-aminobenzamides are significant NF- κ B inhibitors in smooth muscle cells. Although some effects of 4-aminobenzamides are independent of V-ATPase-driven ion trapping (inhibition of NF- κ B nuclear translocation, agent-specific cytotoxicity, PARP cleavage), other effects are dependent on this phenomenon (vacuolization, a component of the cytotoxicity, inhibition of secretion).

We have recently addressed the mechanism of the massive cell vacuolization induced by many organic amines at millimolar concentrations; procainamide, an *N*-substituted 4-aminobenzamide, was the prototype drug used in most assays (Morissette et al., 2004a), but the effect was shared by structurally simpler tertiary amines, such as triethylamine (Fig. 1) or neutral red, a pigmented amine. This reversible drug response consists of the osmotic swelling of the *trans*-Golgi and is prevented by treatment with bafilomycin A1, a V-ATPase inhibitor necessary for the acidification of some in-

tracellular vacuoles (ion trapping of the amines). The subcellular origin of the giant vacuoles was inferred from their perinuclear origin and staining with fluorophore-labeled C5-ceramide. Procainamide induced other effects on cultured smooth muscle cells, including an inhibition of proliferation and migration, but no frank cytotoxicity at 2.5 mM (Morissette et al., 2004a). The vacuolization of the *trans*-Golgi and secretory vesicles has been reported in other cell types in response to chemically disparate amines such as chloroquine and Tris (Peterlik and Kerjaschki, 1979; Back and Soinila, 1996).

In a previous study, procaine at a concentration that produced vacuolization inhibited the mitosis of hepatoma cells or fibroblasts by retarding the late S and G₂ phases of the cell cycle (Henics and Wheatley, 1997). Procaine and procainamide (0.5–1 mM) reportedly demethylate DNA in cultured cells, presumably after direct binding to DNA (Villar-Garea

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ABBREVIATIONS: V-ATPase, vacuolar ATPase; NAPA, *N*-acetyl-procainamide; NAMA, *N*-acetyl-metoclopramide; NF- κ B, nuclear factor- κ B; CHUQ, Centre Hospitalier Universitaire de Québec; GFP, green fluorescent protein; PARP, poly(ADP-ribose)polymerase I; ER, endoplasmic reticulum; RFP, red fluorescent protein.

et al., 2003). A nonapoptotic mitotic arrest has been attributed to this drug effect on cultured cells. Metoclopramide and declopramide (3-chloroprocaïnamide), *N*-substituted 4-aminobenzamides that can be considered substituted procaïnamide derivatives (Fig. 1), exerted further cellular effects, such as apoptosis induction in cultured cells (Liberg et al., 1999; Lindgren et al., 2003), G_2/M cell cycle block (Olsson et al., 2002), and anti-inflammatory properties in vivo and in vitro (Pero et al., 1999). Phase II *N*-acetyl metabolites of procaïnamide-related drug are occurring in vivo (Roden, 2001); *N*-acetyl-procaïnamide (NAPA) is nearly as active as procaïnamide to induce cell vacuolization in smooth muscle cells (Morissette et al., 2004a), but this drug as well as *N*-acetyl-declopramide or *N*-acetyl-metoclopramide (NAMA; Fig. 1) does not induce apoptosis in other cells sensitive to declopramide or metoclopramide. On the other hand, all these drugs except procaïnamide variably inhibit nuclear factor- κ B (NF- κ B) activation in an apparently selective manner in various cell types by preventing I κ B destruction (Liberg et al., 1999; Lindgren et al., 2001, 2003; Olsson et al., 2002). This inhibition could explain, in part, the radiosensi-

tizing and antitumoral effects of these drugs (Baldwin, 2001) and the decreased cytokine secretion in systems where benzamides are used at millimolar or submillimolar concentrations (Pero et al., 1999).

The present experiments aim at documenting whether ion trapping and cell vacuolization have been overlooked and occur at the high concentrations of 4-aminobenzamides that exert cell cycle and cytotoxic effects and whether that form of cell stress determines these effects. A possible mechanism for agent-selective toxicity of trapped amines has been proposed previously (Firestone et al., 1979): the more lipophilic molecules may become detergents upon protonation and concentration in acidic vacuoles, rupturing them and killing the cells rapidly (necrosis). On the other hand, mitochondria are also a site of ion trapping for basic drugs (Siebert et al., 2004; a V-ATPase-independent process), and mitochondrial dysfunction may mediate the cytotoxic response to some of them (Irwin et al., 2002; Enomoto et al., 2004; Menor et al., 2004; Montiel-Duarte et al., 2004). Finally, toxicity, presumably independent from ion trapping, can be produced by some *N*-substituted 4-aminobenzamides that cause poorly repaired DNA strand breaks (Lybak and Pero, 1991).

Primary cultures of rabbit smooth muscle cells, a previously exploited primary cell model (Morissette et al., 2004a), have been used to address the cellular effects of procaïnamide analogs. In addition, the studies were extended to two tumor-derived cell lines to assess the effect of a transformed state on the response to drugs. It was found that massive cell vacuolization and mitotic arrest are predictable and relatively nontoxic effects of 4-aminobenzamides in the millimolar range, but that agent-specific cell death was also recorded via additional mechanisms.

Materials and Methods

Drugs. The synthesis on NAMA is described in the Supplemental data. Human recombinant interleukin-1 β was purchased from R&D Systems (Minneapolis, MN). The remaining drugs were obtained from Sigma-Aldrich (St. Louis, MO).

Cells and Transfection. Several primary cultures of rabbit pulmonary artery were initiated and maintained as described previously (Morissette et al., 2004a). The red fluorescent protein HcRed (transiently transfected as the pHcRed1-N1 vector; BD Biosciences Clontech, Palo Alto, CA), distributed in all cytosolic water, has also been used as a visualization aid in smooth muscle cells, as described previously (Morissette et al., 2004a). The rat Morris 7777 hepatocarcinoma cell line, subclone 7.6 (Guertin et al., 1988; Morissette et al., 2004b), was a gift from Prof. L. Bélanger, [Centre Hospitalier Universitaire de Québec (CHUQ), Québec, QC, Canada]. This cell line is tumorigenic in syngeneic Buffalo rats and secretes the hepatoma marker rat α -fetoprotein. The human fibrosarcoma-derived HT-1080 cell line was a gift from Dr. Eric Petitclerc (Centre de Recherche Hôpital Saint-François d'Assise, Québec, QC, Canada); it is tumorigenic in the chorioallantoic membrane assay (Xu et al., 2001). Both Morris 7777 and HT-1080 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, fresh glutamine, and antibiotics. Both sublines of Morris 7777 and HT-1080 cells that express green fluorescent protein (GFP) were obtained by transfecting cells with the pEGFP-N3 vector (BD Biosciences Clontech) using the X-Gen 500 transfection reagent (MBI Fermentas Inc., Flamborough, ON, Canada) as directed, and selecting stable transfectants after growing the cells for one month in the presence of geneticin (500 μ g/ml; Invitrogen, Carlsbad, CA).

Other Cell-Based Assays. A proliferation assay for smooth muscle cells has been applied as described previously (Morissette et al.,

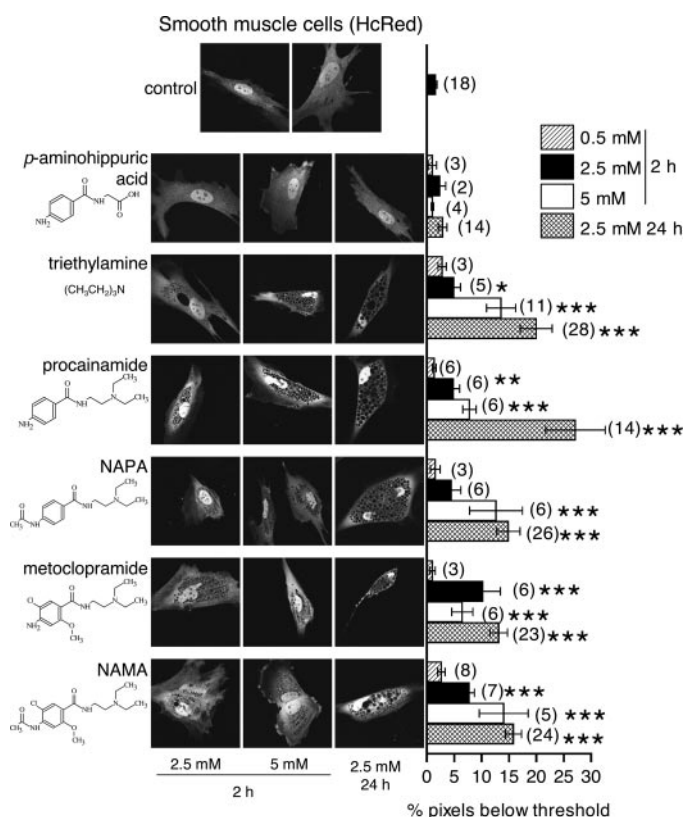


Fig. 1. Vacuolization of smooth muscle cells induced by substituted 4-aminobenzamides and triethylamine as shown in negative in rabbit smooth muscle cells transiently expressing the HcRed fluorescent protein and observed using confocal microscopy. All drugs were added to fetal bovine serum-containing culture media for 2 or 24 h at the indicated concentrations. Square fields have sides of 180 μ m. Representative results of multiple fields observed during at least 2 separate days of experiments. Right, quantification of the vacuolization as a percentage of individual cell surface pixels below a set threshold after image treatment (see *Materials and Methods*). The photographic record for the 0.5 mM concentration of the drugs is not shown (morphologically similar to controls), but the numerical results are shown. Values are the means \pm S.E.M. of the number of evaluated fluorescent cells between parentheses. Values from drug-treated cells were compared with controls using the Mann-Whitney test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

2004a). Poly(ADP-ribose)polymerase I (PARP) is a caspase substrate cleaved in apoptotic cells; immunoblot detection of PARP (116 kDa) and cleaved PARP (89 kDa) was performed in extracts of cells as described previously (Morissette et al., 2004b; based on a polyclonal anti-PARP antibody from Cell Signaling Technologies, Inc.). Immunoblots of α -fetoproteins (based on anti-rat α -fetoprotein polyclonal antibodies, a gift from Prof. L. Bélanger, CHUQ, Québec, QC, Canada) were performed as described previously (Morissette et al., 2004b) and applied to supernatant (secreted) α -fetoprotein (10 μ l/lane) or total cell extracts (25 μ g of protein/lane).

Confocal and Epifluorescence Microscopy. Mitochondrial staining was applied as 0.2 μ M MitoTracker Red CMXRos (Invitrogen) added to the culture medium 30 min before microscopic observation. The Golgi network has been visualized using C5-ceramide-BODIPY FL (Invitrogen) as described previously (Morissette et al., 2004a) by indirect immunofluorescence applied to fixed and permeabilized cells [anti-p230 primary monoclonal antibody; dilution 1:100 (BD Biosciences); secondary antibody goat-anti-mouse IgG-Alexa 488 conjugate, 1:400 (Invitrogen); epifluorescence] or using GFP-golgin-97 [coded by vector GRIP12 (Munro and Nichols, 1999); gift from Dr. Sean Munro, Medical Research Council Laboratory of Molecular Biology, Cambridge, UK]. The endoplasmic reticulum (ER) has been also investigated using an ER-targeted RFP vector coding for a COOH-terminal amino acid sequence SEKDEL ligated downstream to the leader sequence of the surface molecule CD5 (Klee and Pimentel-Muñoz, 2005; gift from Dr. Felipe X. Pimentel-Muñoz, Centro de Investigación del Cáncer, Universidad de Salamanca-CSIS, Salamanca, Spain). The subcellular fluorescence distribution in live cells was observed without fixation using a Bio-Rad (Hercules, CA) 1024 laser beam confocal microscope (40 \times objective with oil immersion; HcRed and MitoTracker: emission, 568 nm; detection, above 585 nm; GFP and C5-ceramide-BODIPY FL: emission, 488 nm; detection, above 510 nm) or epifluorescence microscopy. The vacuolization of fluorescent cells (expressing either HcRed or GFP proteins that are excluded from the vacuoles that appear as dark disks) was quantified as the percentage of individual cell pixels below a set threshold, after increasing the contrast of the picture in a standardized manner for each cell line (Photoshop; Adobe Systems, Mountain View, CA). These numerical values were averaged and compared using nonparametric statistical tests. Three-dimensional reconstructions of cells from series of confocal images were made using the FreeSFP software (<http://www.svi.nl>).

Cytotoxicity Assay. Subconfluent smooth muscle cells plated in 35-mm Petri dishes were maintained in their regular culture medium; test drugs or combinations were added for 24 h. At the end, the medium was removed and centrifuged at 1000 rpm for 5 min. The pellet containing some detached cells was resuspended in the following reaction medium and reintroduced into the Petri dishes. This medium was composed of 1 ml of fresh fluorescein diacetate 12.5 μ g/ml (Sigma-Aldrich) in phosphate-buffered saline diluted from a 5 mg/ml stock in filtered acetone. The cells were incubated for 30 min at 37°C in the dark. Propidium iodide (100 μ l of 0.5 mg/ml) was then added, the dishes were further incubated for 3 to 5 min at room temperature and then observed (epifluorescence 100 \times) without rinsing, using two sets of excitation-emission filters (the red fluorescence indicates propidium iodide uptake by nonviable cells, and the green fluorescence shows the hydrolysis of the fluorescein ester by viable cells). The proportion of nonviable cells (adherent or not) was established by counting. A modification of the same assay was performed on amine-treated, trypsin-EDTA detached cells for the Morris and HT-1080 lines; the green and red fluorescence were quantified using the Elite ESP cytofluorometry apparatus (Beckman Coulter, Fullerton, CA); results were analyzed using the Expo software, version 2.0.

Cell Detachment Assay. A simple assay was applied to tumor-derived cell lines to obtain insight into cell damage. 2.5×10^5 cells were seeded in 35-mm Petri dishes at time -48 h, the fetal bovine serum-containing culture medium (2 ml/dish) was replaced and sup-

plemented by an amine drug at time -24 h, and the detached cells were manually counted at time 0.

Assays for NF- κ B Nuclear Translocation and a Gene under the Control of NF- κ B. Translocation of NF- κ B p65 subunit from the cytosol to the nucleus is studied by indirect immunofluorescence as described previously (Sabourin et al., 2002); the staining intensity of each manually outlined nucleus in the photographic record was quantified as the median pixel intensity (0–255 scale; Photoshop). These numerical values were averaged and compared using nonparametric statistical tests.

The binding of [3 H]Lys-des-Arg 9 -bradykinin to intact, adherent smooth muscle cells was used to evaluate the surface expression of the kinin B $_1$ receptor, a highly regulated protein in immunostimulated cells, as described previously (Sabourin et al., 2002).

Results

Cell Vacuolization Induced by Amine Drugs in Smooth Muscle Cells. Confocal microscopy was used to provide evidence for drug-induced smooth muscle cell vacuolization in cells that express HcRed, because this protein with a cytosolic and nuclear distribution is excluded from giant vacuoles induced by basic agents (Morissette et al., 2004a). Triethylamine and 4-aminobenzamides that can be considered substituted triethylamine (procainamide, NAPA, metoclopramide, NAMA; Fig. 1) all induced the formation of numerous and relatively large dark vacuoles over a 2-h period (Fig. 1). The 0.5 mM level of each drug was inactive, based on cell pixel analysis (Fig. 1 and data not shown), and the threshold for activity was 2.5 mM, except for NAPA (5 mM). For 24-h drug treatments, the 2.5 mM level was active for all these drugs including NAPA, and a more constant vacuole size was obtained (Fig. 1). The intensity of the vacuolization after 4-h treatments with amines was essentially identical to that of 24-h treatments (see control values in subsequent experiments). The compound *p*-aminohippuric acid is a 4-aminobenzamide that is devoid of the tertiary amine function in its side chain (Fig. 1); this acidic analog of procainamide is inactive to induce cell vacuolization (Fig. 1).

Morphologic Analysis of Responses to Amines in Smooth Muscle Cells and Their Inhibition by Bafilomycin A1. Figures 2 and 3 depict the morphological responses to concentrated amine drugs in smooth muscle cells, a large cell type well adapted for this purpose. A three-dimensional reconstitution of smooth muscle cells treated or not with the low-toxicity amine procainamide is shown in Fig. 2 (left column). HcRed filled the space in a control cell, but procainamide (2.5 mM, 4 h) induced the formation of apparently rigid spherical vacuoles that excluded the fluorescent protein and imprinted on the nucleus.

Triethylamine, procainamide, and metoclopramide are amines of variable structural complexity that were included in the morphological analysis of smooth muscle cell vacuolization. As has been shown qualitatively for procainamide (Morissette et al., 2004a), the vacuolization induced by this amine, triethylamine, or metoclopramide (2.5 mM, 4 h) was largely prevented by concomitant treatment with the V-ATPase inhibitor bafilomycin A1 (120 nM; Fig. 3; statistical analysis of numerized cell pictures, Fig. 4). At 4 h of observation time, many cells exposed to metoclopramide exhibited morphological signs of toxicity, such as rounding (Fig. 3; this applied to 24-h treatments and to the drug NAMA as well; Fig. 1); bafilomycin variably reduced those changes (Fig. 3).

The identity of the amine-induced giant vacuoles has been further investigated in cells treated with the Golgi marker C5-ceramide. Ribbons clustered adjacent to the nucleus were labeled with this fluorescent compound in control cells; specific staining of the giant vacuole membranes is observed in cells treated with triethylamine, procainamide, or metoclopramide (Fig. 3). The relationship of the giant vacuoles with the Golgi network has been further studied using two *trans*-Golgi markers, p230 (indirect immunofluorescence, Fig. 3) and golgin-97 (GFP fusion protein, epifluorescence, Fig. 2,

right column) in smooth muscle cells. The ribbon-like staining for either protein in control cells was transformed by a 4-h treatment with triethylamine, procainamide, or metoclopramide into a pattern in which a fraction of contiguous giant vacuoles was labeled (microscopy data not shown for triethylamine and metoclopramide with golgin-97). Amine-induced vacuolization did not arise from mitochondria, in that the vacuoles are not continuously lined with the fluorophore MitoTracker, but mitochondrial morphology may be affected by amines: triethylamine dilated some, and procainamide or

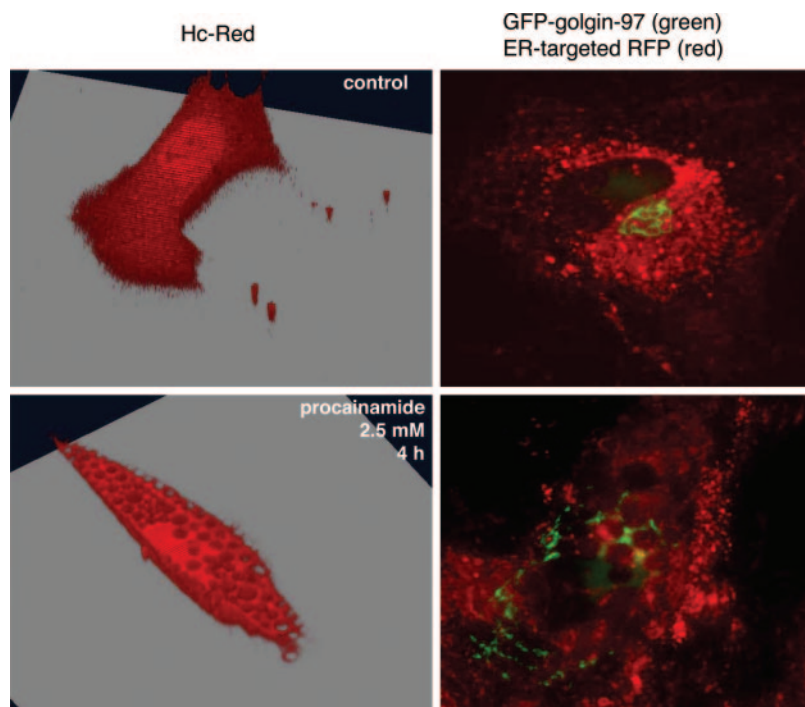


Fig. 2. Morphologic studies of procainamide-treated smooth muscle cells. Left column, three-dimensional reconstitution of smooth muscle cells expressing HcRed: effect of procainamide (2.5 mM, 4 h). Right column, dual epifluorescence imaging (600 \times) of GFP-golgin-97 (green) and ER-targeted-RFP (red) in control or procainamide-treated smooth muscle cells.

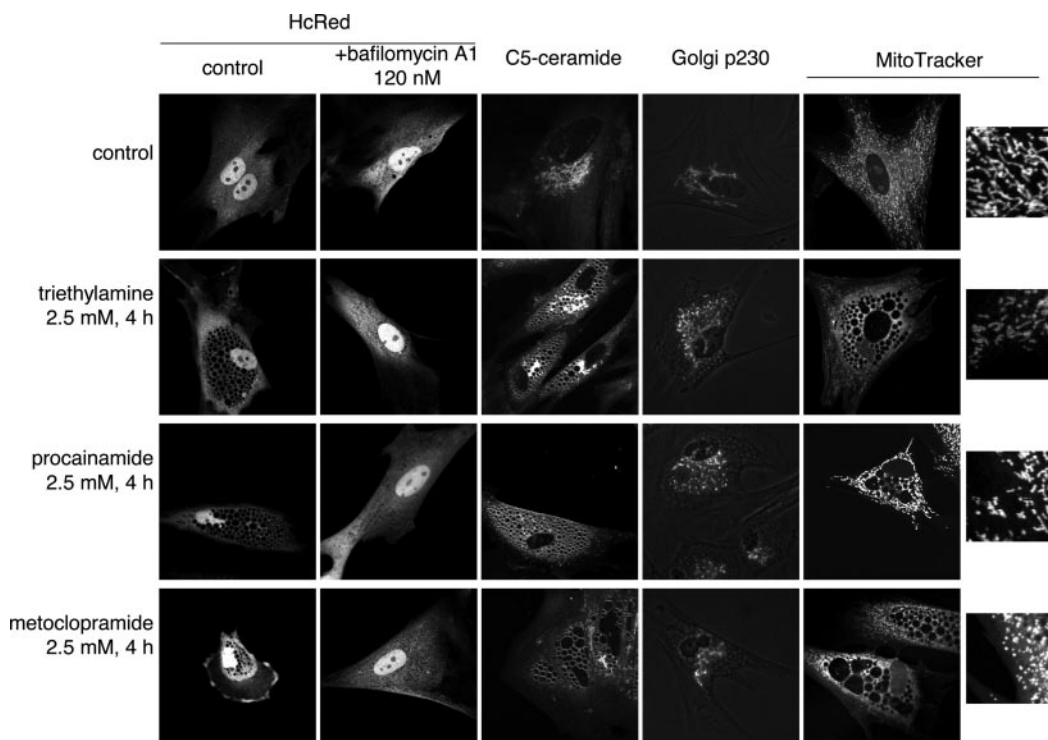


Fig. 3. Effect of 4-h treatments with some amines on smooth muscle cell morphology as assessed by various fluorophores. Golgi 230 immunofluorescence was recorded using a dual imaging mode (phase contrast and epifluorescence, 400 \times); the other frames are confocal micrographs presented as in Fig. 1 (close-up views provided for MitoTracker imaging). See *Results* for further description.

metoclopramide rather condensed them (better seen in the periphery of the cells or in close-up views, Fig. 3). ER-targeted RFP exhibited a granular cytosolic distribution that also remained independent from procainamide-induced giant vacuoles (Fig. 2, right column; this also applied to cells treated with triethylamine or metoclopramide, data not shown).

Effects of Amine Drugs on Smooth Muscle Cell Proliferation and Viability. The inhibition of proliferation is a typical response to procainamide-related drugs (see Introduction). A study conducted over 48 h showed a complete inhibition of proliferation applicable to all amine drugs applied at 2.5 mM, including triethylamine (Fig. 5A, as previously observed for procainamide, Morissette et al., 2004a). The number of recovered cells was inferior to the number of seeded cells (5×10^4) for some of the drug treatments (NAPA, metoclopramide, NAMA). The V-ATPase inhibitor did not prevent the mitotic arrest induced by the noncytotoxic amines procainamide or triethylamine (2.5 mM, Fig. 5B); however, bafilomycin A1 itself produced a complete inhibition of proliferation.

A cytotoxicity assay applied to the smooth muscle cells showed that actinomycin D (400 nM), a proven inducer of apoptosis in these cells (Morissette et al., 2004a), determined a cytotoxic response (24 h, sample photographic record, Fig. 6A; statistical analysis, Fig. 6B). Bafilomycin A1 produced a small cytotoxicity that became synergistic with that caused by actinomycin D when the two drugs were combined. The 2.5 mM concentration level of triethylamine, procainamide, or metoclopramide exerted small cytotoxic effects of increasing intensity in this order over 24 h. At a higher level (5 mM), triethylamine and procainamide exhibited more frequent cell death, and this was partially prevented by bafilomycin A1 cotreatment (Fig. 6B). Metoclopramide (5 mM) exerted an

even more frequent cytotoxicity that was not relieved by bafilomycin cotreatment.

Effects of Amine Drugs on NF- κ B and an NF- κ B-Regulated Gene in Smooth Muscle Cell. In serum-starved smooth muscle cells, the p65 subunit of NF- κ B had a mainly cytosolic localization (Fig. 7A). Interleukin-1 β treatment (1 h) strongly concentrated p65 into the nuclei in most cells (Fig. 7A; statistics on median nuclear pixel intensity in Fig. 7B). At 2.5 mM, agents that induce vacuolization produced no effect on the baseline nuclear fluorescence (triethylamine, procainamide, metoclopramide) or a slight significant inhibition (NAPA) or stimulation of this baseline (NAMA) (Fig. 7, A and B; the sample photographic record also shows p65-excluding cytosolic vacuoles). The amine drugs (applied 2 h before staining) exerted variable interactions with the effect of interleukin-1 β (applied 1 h before staining): triethylamine and procainamide did not change the strong activating effect of the cytokine, but NAPA, metoclopramide, and its amide NAMA significantly reduced it (Fig. 7B). Although bafilomycin A1 significantly increased

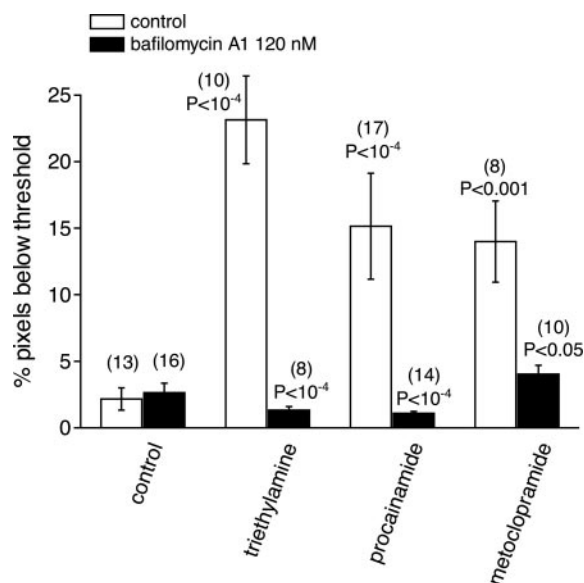


Fig. 4. Numerization and analysis of the effect of bafilomycin A1 on the vacuolization induced by triethylamine, procainamide, or metoclopramide (2.5 mM, 4-h concurrent treatments) in smooth muscle cells expressing HcRed (sample microscopic record in Fig. 3). *P* values above open bars are the results of the comparison between the control without bafilomycin or amine. *P* values above closed bars indicate differences recorded in the presence of bafilomycin for each drug treatment (Mann-Whitney *U* test with the open bar next to it).

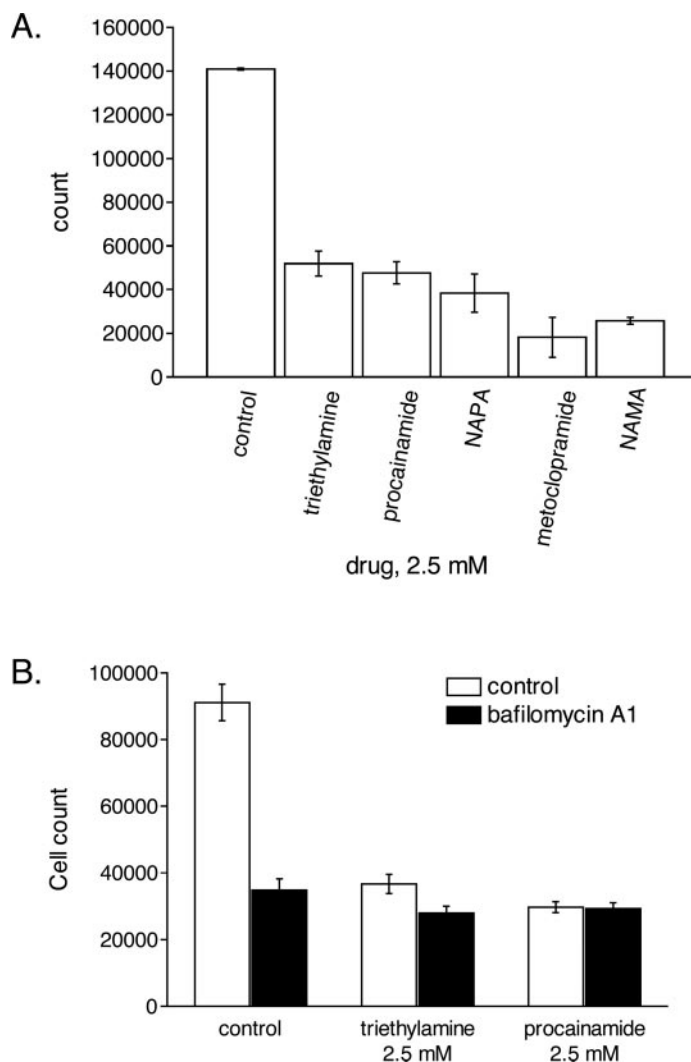


Fig. 5. A, effect of basic drugs on smooth muscle cell proliferation. Petri dishes were seeded at time 0 with 5×10^4 cells; drug treatments were initiated at time 24 h and cell counts were performed at 72 h. Values are the means \pm S.E.M. of three determinations. B, effect of bafilomycin A1 concurrent treatment on triethylamine- or procainamide-induced mitotic arrest in smooth muscle cells. Presentation as in A.

the p65 nuclear content in interleukin-1 β -cotreated smooth muscle cells, metoclopramide was still effective enough to significantly reduce the nuclear staining intensity when combined with bafilomycin (Fig. 7C). Thus, this effect is not V-ATPase dependent; rather, it is consistent with the reported inhibitory effect of highly substituted 4-aminobenzamides on I κ B kinase (see Introduction). The kinin B₁ receptor is expressed under the control of NF- κ B in rabbit arterial smooth muscle cells (Sabourin et al., 2002). A binding assay based on a saturating concentration of the agonist radioligand [³H]Lys-des-Arg⁹-bradykinin (1 nM) showed that a 4-h treatment of the smooth muscle cells with procainamide or metoclopramide (2.5 mM) failed to up-regulate the B₁ receptors. On the contrary, metoclopramide significantly decreased the baseline expression of the cell surface binding sites. The documented stimulatory effect of interleukin-1 β on receptor abundance is reduced by concomitant treatment with either amine (Fig. 7D). Bafilomycin A1 treatment of cells did not inhibit the interleukin-1 stimulation of B₁ receptor expression, but partially and significantly reversed the inhibitory effect of procainamide on this process, not that of metoclopramide (Fig. 7D).

The investigations have been extended to the tumor-derived Morris hepatoma and HT-1080 fibrosarcoma cells expressing GFP to probe whether a transformed phenotype sensitizes the cells to cytotoxicity induced by 4-aminobenzamides.

Effect of Amines on Morris 7777 Cells. As for the smooth muscle cell, triethylamine, procainamide, NAPA, metoclopramide, and NAMA induced the vacuolization of Morris cells in 2 h (Fig. 8). The threshold active concentration was 2.5 mM (except for triethylamine at 5 mM). However, a 4-h treatment with 2.5 mM triethylamine was significantly active (Fig. 9A, comparatively less than the same amine at 15 mM or than metoclopramide or procainamide at 2.5 mM). Figure 9A also shows that the vacuolization response was

prevented by concurrent bafilomycin A1 treatment in Morris cells. Independently of vacuolization, metoclopramide frequently induced “blebbing” and rounding in Morris cells (Figs. 8 and 9). As in the smooth muscle cells, MitoTracker did not label the giant vacuoles induced by triethylamine (15 mM) or metoclopramide (2.5 mM) that might, nevertheless, modify the appearance of mitochondria in Morris cells (Fig. 9B).

The hepatoma-derived Morris cell line has the capacity to secrete large quantities of α -fetoprotein (Morissette et al., 2004b), which is an opportunity to test the effect of amines that induce vacuolization on the secretory pathway (Fig. 10). In both the cell extract and supernatant of control Morris cells, mature ~70 kDa α -fetoprotein is abundant (Fig. 10A). The amines triethylamine, procainamide, and metoclopramide at 5 mM reduced the supernatant protein rather than the intracellular α -fetoprotein contents, as if the secretion were impaired. This effect compares well with that of brefeldin A, a known Golgi-disrupting agent (Doms et al., 1989), but not with that the protein synthesis inhibitor anisomycin or the glycosylation inhibitor tunicamycin, which reduced both intra- and extracellular α -fetoprotein (and lowered the apparent molecular weight of the glycoprotein for tunicamycin). A detailed concentration-effect study for triethylamine and procainamide showed that these agents inhibit α -fetoprotein secretion precisely at concentrations that produce vacuolization (compare Fig. 10B with Fig. 8). Concurrent treatment of the cells with bafilomycin A1 partially restored the secretion of α -fetoprotein in cells treated with the amines triethylamine or procainamide but not those treated with brefeldin (Fig. 10C), also supporting the idea that the vacuolization induced by the former agents is associated with the impaired secretion process.

The cytofluorometric assay for drug-induced Morris cell damage is illustrated in Fig. 11. These cells are prone to detachment when treated for 24 h with many drugs, and both

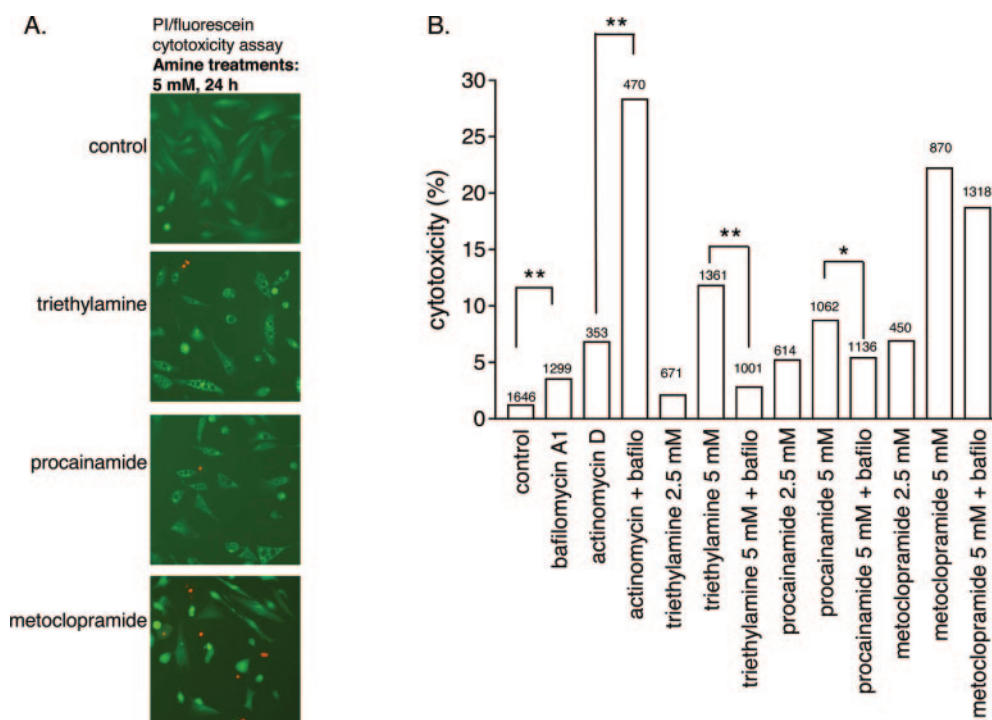
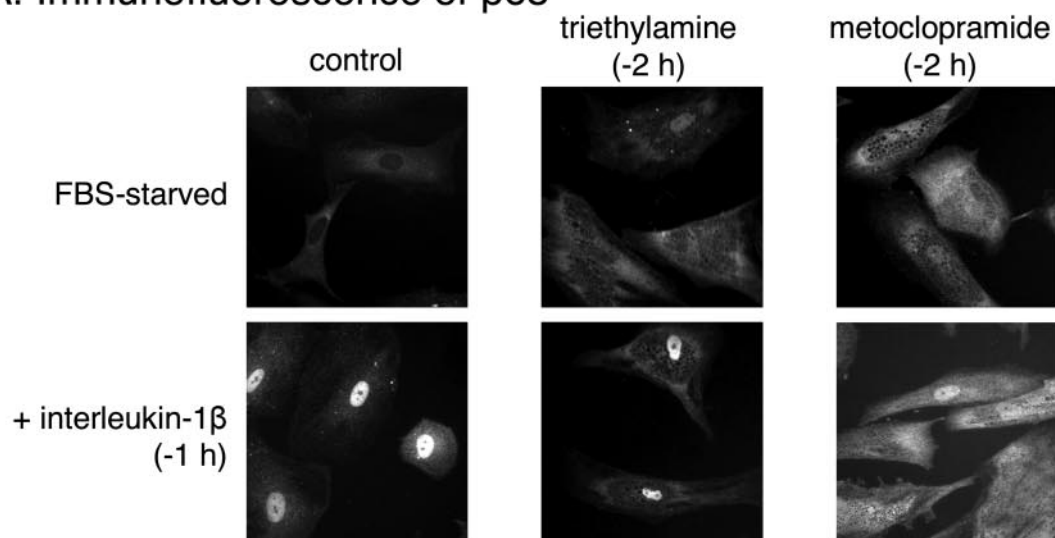
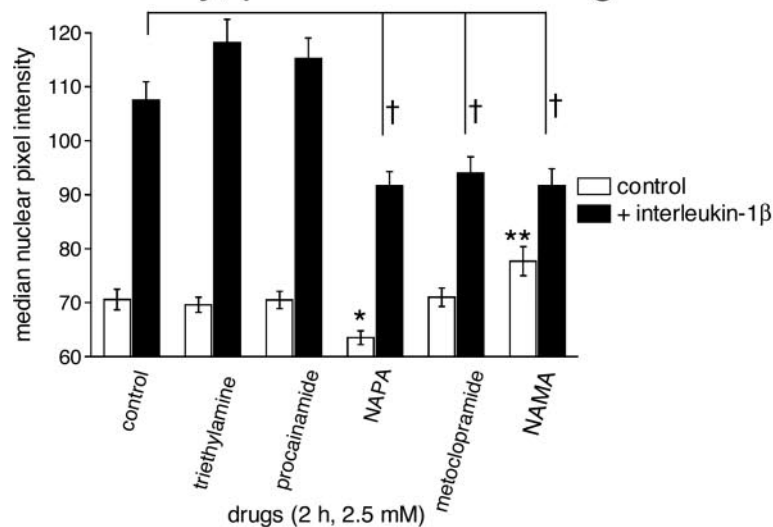


Fig. 6. Cytotoxicity assay applied to smooth muscle cells. Cells were exposed to the indicated drugs or combinations for 24 h (400 nM actinomycin D, 120 nM bafilomycin A1). A, illustration of the cytotoxicity assay based on two fluorophores (100 \times). B, the values are the proportions (%) of nonviable (red) cells. The total cell count is indicated above each bar. The effect of bafilomycin on cytotoxicity was tested (χ^2 test): *, $P < 0.01$; **, $P < 0.001$.

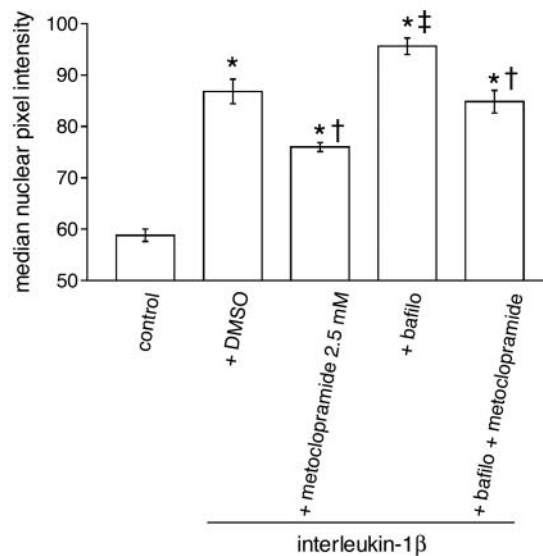
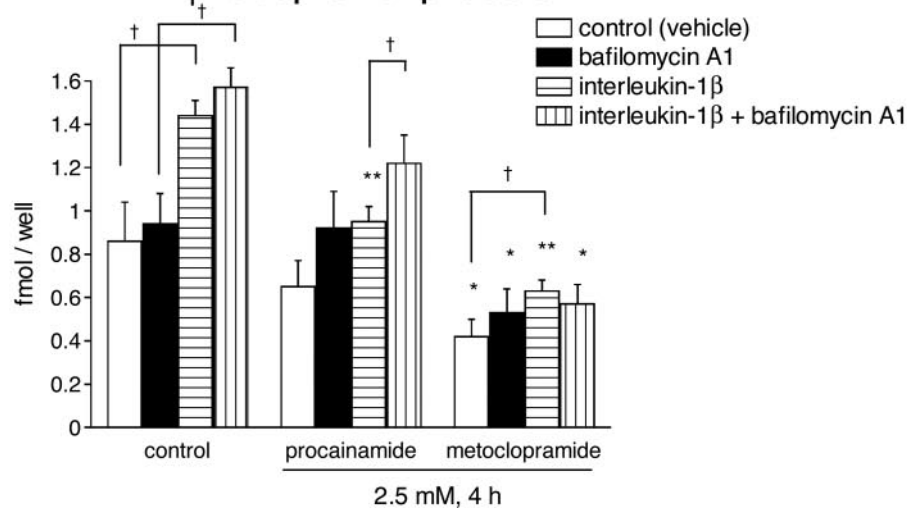
A. Immunofluorescence of p65



B. Intensity, p65 nuclear staining



C.

D. Kinin B₁ receptor expression

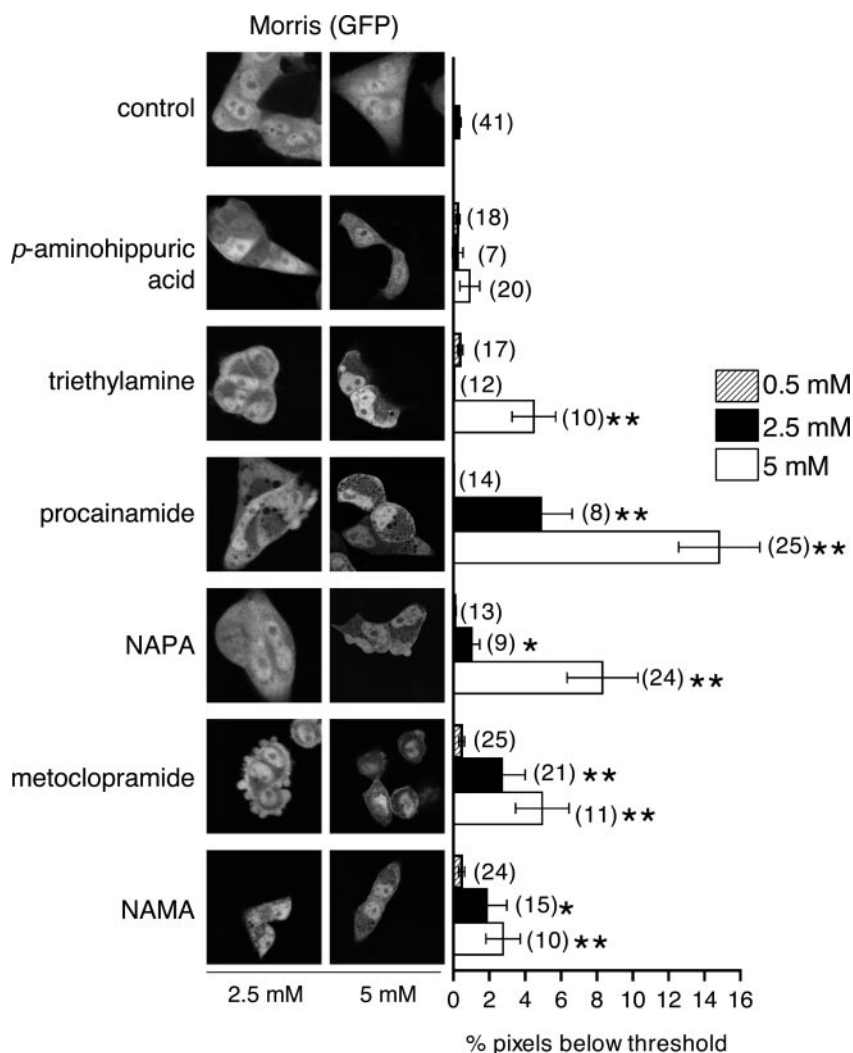


Fig. 8. Effect of 2-h treatments with substituted 4-aminobenzamides (including *p*-aminohippuric acid) or triethylamine on Morris 7777 cells stably expressing GFP and observed using confocal microscopy. All drugs were added to fetal bovine serum-containing culture media. Square fields have sides of 60 μ m. Representative results of multiple fields observed during at least 2 separate days of experiments. Right, quantification of the vacuolization as a percent of individual cell surface pixels below a set threshold after image treatment (see *Materials and Methods*). Values are the means \pm S.E.M. of the number of evaluated fluorescent cells between parentheses. The photographic record for the 0.5 mM concentration of the drugs is not shown (morphologically similar to controls), but the numerical results are shown. Values from drug-treated cells were compared with controls using the Mann-Whitney *U* test (*, $P < 0.01$; **, $P < 0.001$).

detached and attached cells were included in the analysis. Toxicity is defined as the exclusion from the area of healthy cells (rectangle E in the recordings, Fig. 11A, representing cells that have not taken up propidium iodide but that have efficiently hydrolyzed fluorescein diacetate to form the green fluorophore). Bafilomycin A1 treatment (120 nM) exerted a small toxic effect that was apparently additive to that of other treatments. Actinomycin D induced a large toxic response that was not relieved by bafilomycin A1. The cytotoxic effects of triethylamine and procainamide (2.5–5 mM) was comparatively small, but concentration-dependent. Metoclopramide was much more toxic than procainamide at each

concentration. Bafilomycin cotreatment inhibited only the toxic effect of 5 mM procainamide (Fig. 11B).

PARP cleavage occurs more intensely in Morris cells treated with metoclopramide (2.5 mM, 24 h) than in those treated with triethylamine, procainamide, or NAPA (Fig. 12, top). Bafilomycin A1 (120 nM) is not active in this respect. Actinomycin D was used as a positive control for apoptosis induction. Bafilomycin A1 cotreatment did not prevent the effect of metoclopramide on PARP cleavage (Fig. 12).

Effect of Amines on HT-1080 Cells. Essential verifications were performed using this alternate tumor-derived line of human origin. Thus, procainamide, metoclopramide, and

Fig. 7. Effect of interleukin-1 β (5 ng/ml) and amine drugs (2.5 mM) on the nuclear translocation of NF- κ B p65 in smooth muscle cells. A, sample photographic record. B, median pixel intensity in the nuclei (applied to at least 90 cells per condition during 2 separate days of experiments). The intensity values recorded in the presence of interleukin-1 β are significantly different from that recorded in the absence of the cytokine, regardless of drugs (comparison of neighboring closed and open bars, $P < 0.001$, Mann-Whitney *U* test). Two of the amine drugs produced intensity values significantly different from the controls without interleukin-1 β (*, $P < 0.05$; **, $P < 0.005$). In addition, NAPA, metoclopramide and NAMA reduced the effect of interleukin-1 β (\ddagger , $P < 10^{-4}$). C, the inhibitory effect of metoclopramide on interleukin-1-induced nuclear translocation of p65 is not prevented by bafilomycin A1 cotreatment. Values are the means \pm S.E.M. of 49 to 176 determinations. Values in the control group were compared with those from those of treatments that included interleukin-1 β (5 ng/ml); *, $P < 10^{-4}$. Further comparison for the effect of adding metoclopramide cotreatments were performed (\ddagger , $P < 0.001$). Bafilomycin A1 cotreatment significantly increased the intensity of the response in interleukin-1 β -treated cells (\ddagger , $P < 10^{-4}$). D, effect of amine drugs on the expression of a NF- κ B-dependent gene in fetal bovine serum-starved smooth muscle cells, the kinin B $_1$ receptor, as established using the binding of 1 nM [3 H]Lys-des-Arg 9 -bradykinin 4 h after treatments with amines, concomitantly or not with interleukin-1 β (5 ng/ml) and/or bafilomycin A1 (120 nM). Results are the means \pm S.E.M. of four to five determinations composed of duplicate values. Values in the control groups were compared with those from amine-treated cells with the same cotreatment (interleukin-1 β 5 ng/ml, and/or bafilomycin A1 120 nM; *, $P < 0.05$; **, $P < 0.01$ by Mann-Whitney test). Further comparison for the effect of cotreatments were performed (\ddagger , $P < 0.05$).

triethylamine vacuolized HT-1080 cells in 2 h (triethylamine less effectively at 2.5 mM, but well at 15 mM; Fig. 13); metoclopramide produced extensive blebbing; and concurrent treatment with bafilomycin A1 abated the vacuolization induced by all agents but not the blebbing induced by metoclopramide in many cells (Fig. 13). A certain level of perinuclear vacuolization in some control cells was the result of the arrangement of mitochondria, which often radiate from the cell center (compare MitoTracker staining with GFP in Fig. 13B). The large vacuoles remained independent from the mitochondria in cells in which vacuolization was induced by an amine (Fig. 13B). The cytofluorometric assay for toxicity applied in a manner identical to that for Morris cells showed that actinomycin D is less toxic for HT-1080 cells than metoclopramide (2.5 mM), whereas triethylamine- and procainamide-induced toxicity was minimal (Fig. 14). Therefore,

PARP cleavage induced by metoclopramide in HT-1080 cells was more complete than that induced by actinomycin D, and triethylamine and procainamide were much less active (Fig. 12). Bafilomycin A1 cotreatment reduced the small toxicity of procainamide in a sizeable manner and that of metoclopramide to a smaller extent and exerted a small direct effect that was additive to that of actinomycin or triethylamine (Fig. 14). Although bafilomycin A1 cotreatment slightly decreased metoclopramide-induced toxicity in HT-1080 cells (Fig. 14B), it did not reduce metoclopramide-induced PARP cleavage in this line (Fig. 12). In fact, the bafilomycin-metoclopramide combination seemed to accelerate PARP degradation into undetectable cleaved fragments, in that the abundance of both nondegraded (116 kDa) and degraded PARP (89 kDa) was low in repeated experiments (Fig. 12).

The special sensitivity of HT-1080 cells to metoclopramide

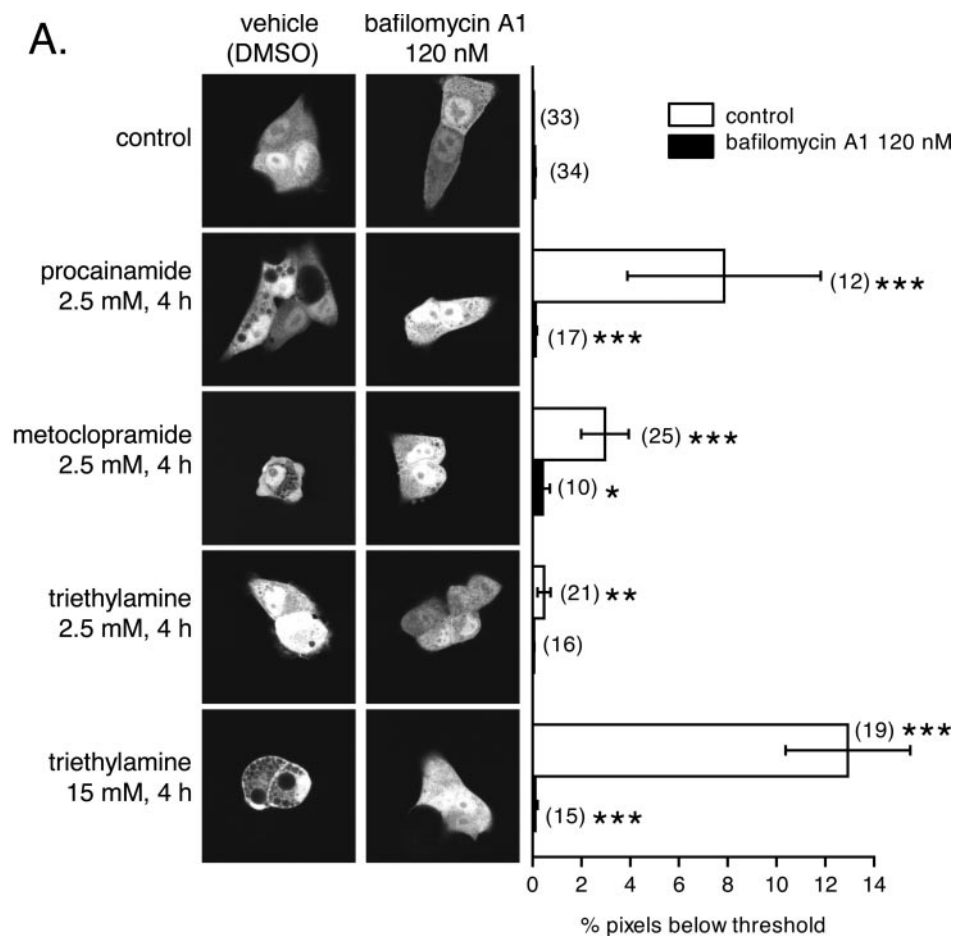
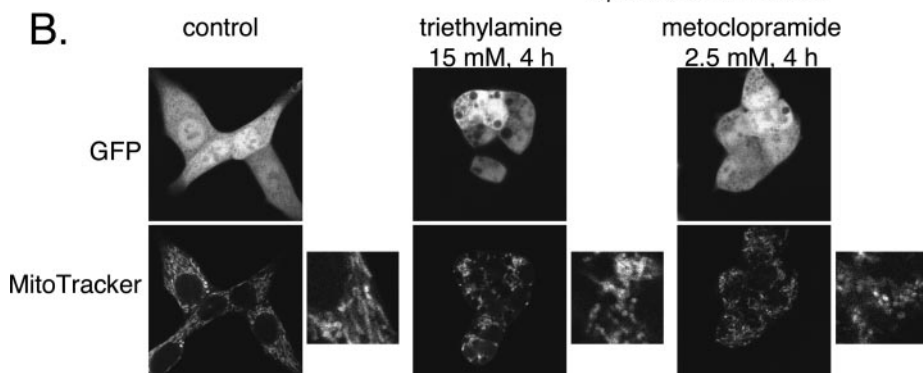


Fig. 9. Effect of various drugs on Morris 7777 cells. **A**, the vacuolization induced by amines is inhibited by concurrent treatment with bafilomycin A1. Presentation as in Fig. 8. *P* values for the open bars refer to a Mann-Whitney *U* test comparing the corresponding group with the control; the *P* values for each solid bar (effect of bafilomycin) are Mann-Whitney *U* comparisons with cells treated with the same amine (the open bar next to it). **B**, combined imaging of MitoTracker and GFP in amine-treated cells (close-up views provided for MitoTracker imaging).



was further documented in a simple detachment assay that integrates various forms of cell damage (Fig. 15). Relative to the Morris cells, for which metoclopramide is only 1.3-fold more active than procainamide in terms of cell detachment, metoclopramide is 5.1-fold more potent than procainamide in HT-1080 cells.

Discussion

Drug effects recorded in the present study occur at high concentrations, higher than the clinical antiarrhythmic concentration of procainamide (Roden, 2001) or than those binding G protein-coupled receptors in the gastroenterology applications of metoclopramide. Thus, the present results may

be of toxicological interest. However, experimental oncology applications and anti-inflammatory properties of 4-aminobenzamides rely on drug concentrations $\geq 500 \mu\text{M}$ (Pero et al., 1999; Olsson et al., 2002; Villar-Garea et al., 2003), a range at which the vacuolization response to organic amines apply to many cell types (Henics and Wheatley, 1997; Morissette et al., 2004a; current study). Whether specific transport or extrusion modulate the accessibility of the amine drugs to intracellular targets is not excluded, but the protonated tertiary amines are in equilibrium with an uncharged form for which the simple diffusion into cells is likely to be effective. According to the literature, procainamide-related 4-aminobenzamide drugs (Fig. 1) exhibit pK_a values between 9 and 10, and that of triethylamine is estimated at 10.7 to 11.0.

The 4-aminobenzamides induce massive smooth muscle cell vacuolization in V-ATPase-dependent manner (Fig. 3). The structural basis of this effect is the presence of triethylamine, because this amine and the series of 4-aminobenzamides that includes triethylamine (Fig. 1) are nearly equipotent to induce *trans*-Golgi swelling in smooth muscle cells, based on the formation of giant vacuoles that exclude cytosolic proteins such as HcRed and whose membranes are labeled with C5-ceramide (Fig. 3), a recognized marker of the

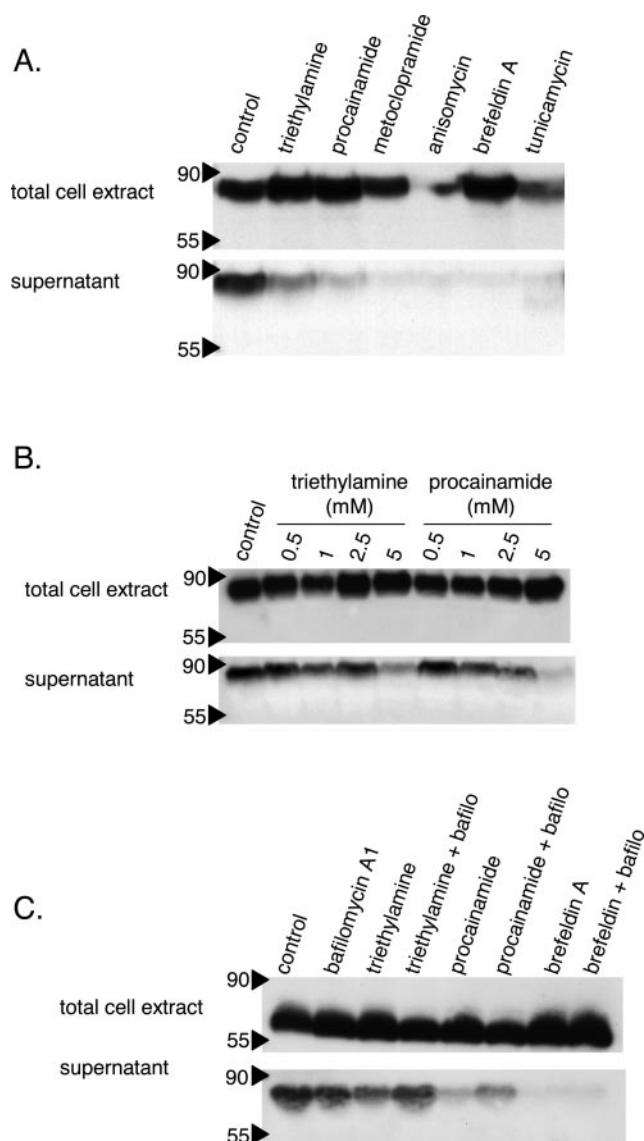


Fig. 10. A, progression of α -fetoprotein in the secretory pathway of Morris cells as modified by amines (5 mM, 4 h) and other drugs (10 μM anisomycin, 18 μM brefeldin A, 25 $\mu\text{g/ml}$ tunicamycin). The cells were washed with fresh medium containing the same drugs in the middle of incubation, so the α -fetoprotein has been secreted into the supernatants during the second half of the 4-h treatment period. B, concentration-effect relationship for the effect of triethylamine and procainamide on α -fetoprotein secretion. C, concurrent treatment with bafilomycin A1 (120 nM) partially restores the secretion of α -fetoprotein inhibited by triethylamine or procainamide (5 mM) but not by brefeldin A.

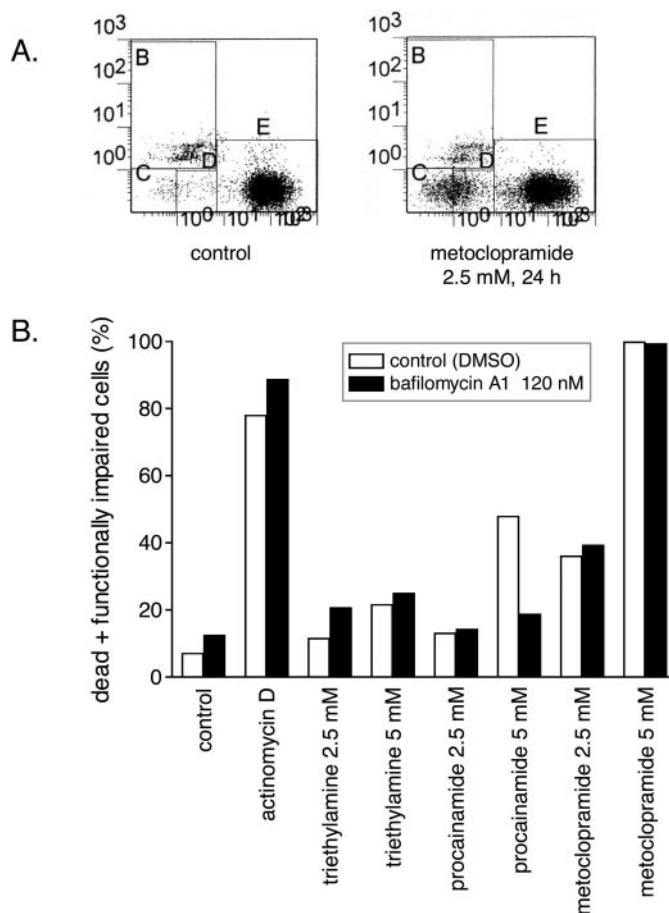


Fig. 11. Cytofluorometric assessment of cell damage induced by amines in untransfected Morris 7777 cells. A, Sample distribution records for 10^4 cells from control or metoclopramide-treated flasks (abscissa, log green fluorescence from fluorescein; ordinate, log of red fluorescence from propidium iodide). Intact and functional cells are in the rectangle E. The proportion of damaged or functionally impaired cells (summed rectangles B, C, and D) is presented in B for each treatment (24 h; drug concentrations: 400 nM actinomycin D, 120 nM bafilomycin A1, or as indicated).

Golgi network and downstream secretory pathway (Pagano et al., 1991; Ktistakis et al., 1995). Both golgin-97 and p230 are peripheral membrane proteins associated with the cytosolic face of the *trans*-Golgi network (Kjer-Nielsen et al.,

1999; Munro and Nichols, 1999); the corresponding cell labeling in smooth muscle cells concerns a minority of contiguous giant vacuoles in amine-treated cells (Figs. 2 and 3). This may be caused by the distribution of these proteins to a subcompartment of the *trans*-Golgi network, or by the vacuolization of other organelles that express V-ATPase further down the secretory pathway. Other laboratories have found evidence of *trans*-Golgi swelling when cells were treated with various amines, such as chloroquine and Tris (Peterlik and Kerjaschki, 1979; Back and Soinila, 1996). However, the bafilomycin-sensitive vacuolization of HeLa cells induced by hydroxychloroquine (a substituted triethylamine) was attributed rather to the swelling of lysosomes (Boya et al., 2003), an interpretation that may be partly applicable to smooth muscle cells, because lysosomes are *trans*-Golgi-derived organelles that express V-ATPase. In Morris 7777 cells that abundantly secrete α -fetoprotein, the amines triethylamine, procainamide, and metoclopramide inhibited the secretion of the protein but not its synthesis, as judged by the intracellular contents (Fig. 10), consistent with an impairment of the secretory pathway distal to the ER. This effect is similar to

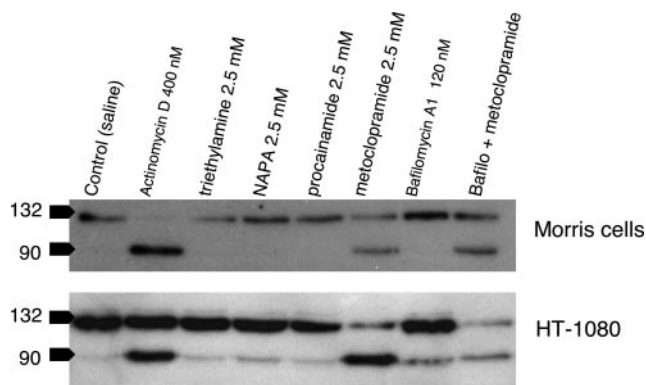


Fig. 12. Immunoblot of PARP and cleaved PARP in the extract of both attached and detached Morris or HT-1080 cells incubated for 24 h with the indicated drug or drug combination. Representative results of two to three experiments.

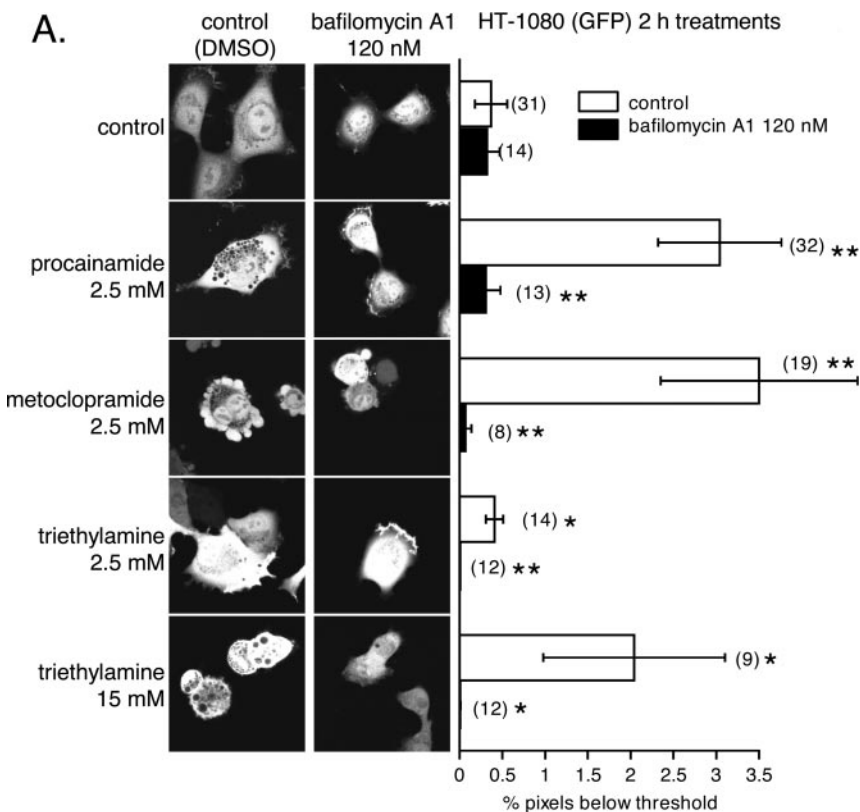
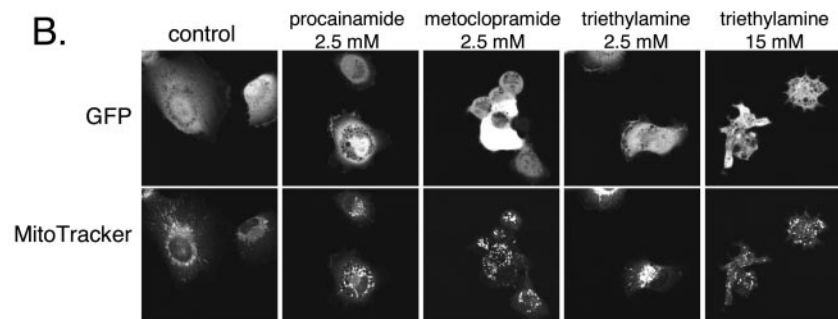


Fig. 13. A, effect of 2-h treatments with amines HT-1080 cells stably expressing GFP and observed using confocal microscopy and effect of bafilomycin A1 on the vacuolization process. All drugs were added to fetal bovine serum-containing culture media. Square fields have sides of 90 μ m. Representative results of multiple fields observed during at least 2 separate days of experiments. Right, quantification of the vacuolization as a percent of individual cell pixels below a set threshold after image treatment (see *Materials and Methods*). Values are the means \pm S.E.M. of the number of evaluated fluorescent cells between parentheses. Values were compared using the Mann-Whitney test (*, $P < 0.01$; **, $P < 0.001$). P values for the open bars refer to comparisons of the corresponding group with the control; the P values for the solid bars (effect of bafilomycin) are Mann-Whitney comparisons with cells treated with the same amine (the open bar next to it). B, combined imaging of MitoTracker and GFP in amine-treated HT-1080 cells (2-h drug treatment).



the Golgi-disrupting drug brefeldin A, but the antiseecretory effect of amines is partially reversed by V-ATPase inhibition, unlike that of brefeldin, supporting the ideas that the formation of the giant vacuoles impairs the trafficking in the secretory pathway and that this effect is more important than that of V-ATPase inhibition.

Mitotic arrest is also a response common to triethylamine and 4-aminobenzamides substituted with triethylamine. The concentration-effect relationship for procainamide-induced mitotic arrest in smooth muscle cell is similar to that of procainamide-induced vacuolization (Morissette et al., 2004a). Bafilomycin A1 itself induced a complete mitotic arrest in smooth muscle cells (Fig. 4) and in several tumor-derived cell lines in a recent study (IC_{50} well under 100 nM; McSheehy et al., 2003). The latter report documents that this effect was not apoptotic and involved a block in S and G_2/M phases. An undetermined step of cell division may require the acidification of a cell compartment by the V-ATPase, and the concentrated amine drugs may reproduce the effect of bafilomycin by directly buffering acidity in this compartment. Whether direct DNA binding explains the nonapoptotic mitotic arrest induced by procainamide-related drugs (Villar-Garea et al., 2003) is unclear, because the response applies to the simple amine triethylamine or to the unrelated molecule bafilomycin A1. It has been reported that the inhibition of the spontaneous mitosis-associated Golgi fragmentation in NRK cells causes a G_2 phase arrest (Sütterlin et al., 2002). Another line of explanation for the mitotic arrest involves iron trans-

port restriction in cells that lack the V-ATPase function (Saurin et al., 1996).

The very high concentration of 5 mM triethylamine or procainamide in smooth muscle cells caused some cytotoxicity that was reversed by bafilomycin A1 (Fig. 6), and this also applied to Morris (procainamide, 5 mM, Fig. 11) and HT-1080 cells (procainamide, 2.5 mM, Fig. 14). This suggests that the concentration of simple amines into organelles possessing the V-ATPase leads to catastrophic vacuolar rupture (as postulated by Firestone et al., 1979). The presence of HcRed within the giant vacuoles induced by at least one of the toxic amines, NAMA (2.5 mM), may support this mechanism (Fig. 1). A series of long-chain *N*-alkylated imino sugars (tertiary amines) are toxic to cells essentially by disrupting membranes in a manner highly dependent on the alkyl chain length (Mellor et al., 2003). These agents, as well as some of the molecules in the present series (Fig. 1), may be concentrated in acidic cell vacuoles by V-ATPase-driven ion trapping and may precipitate their rupture.

Confirming previous reports based on leukemia-derived cell lines (Liberg et al., 1999; Olsson et al., 2002), we found that the toxic compound metoclopramide leads to apoptotic

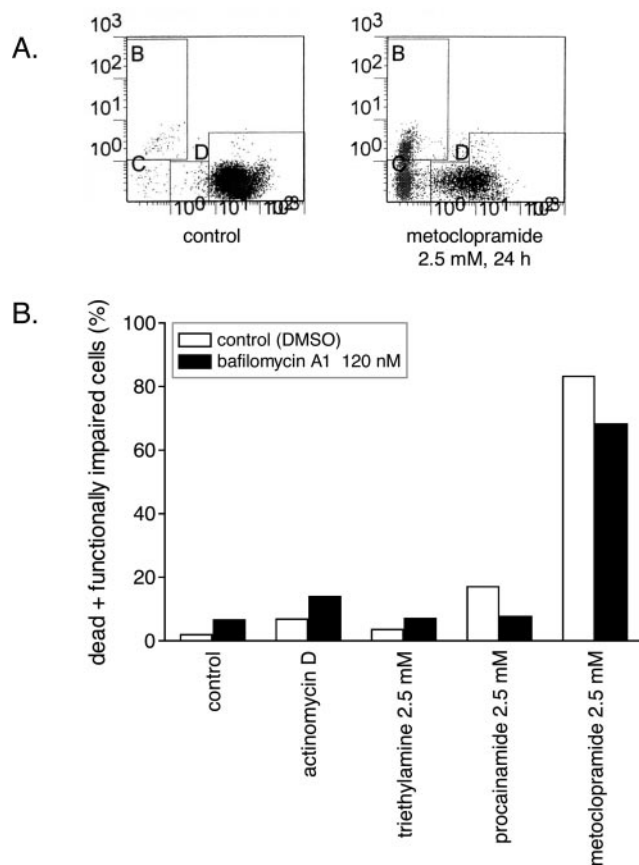


Fig. 14. Cytofluorometric assessment of cell damage induced by amines in untransfected HT-1080 cells. A, sample distribution records for 10^4 cells in control or metoclopramide-treated flasks. B, effect of 24-h drug treatments. Presentation as in Fig. 11.

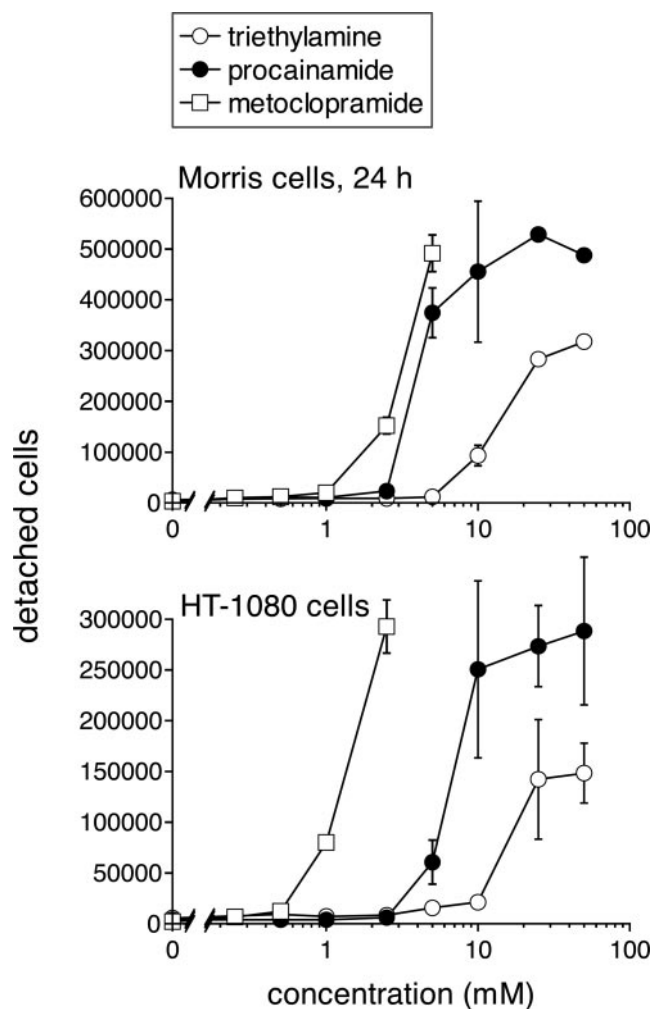


Fig. 15. Cell detachment assay applied to the two tumor-derived cell lines. Values are the means \pm S.E.M. of two separate experiments (2.5×10^5 cells were seeded in 35-mm Petri dishes at time -48 h, the indicated drug was applied at time -24 h, and the detached cells were counted at time 0).

cell death in two tumor-derived cell lines (Fig. 12, PARP cleavage assay, a distal apoptotic event). Halogen substitution of the drug aromatic cycle may be necessary for this effect, because it is not produced by procainamide or NAPA (Fig. 12) but is reportedly shared with declopramide (Olsson et al., 2002). Primary smooth muscle cells were rather refractory to the cytotoxic action of aminobenzamides, supporting a selectivity for cell types, but metoclopramide had some activity at 5 mM. Furthermore, metoclopramide-induced cytotoxicity is not importantly relieved by bafilomycin in any of the three tested cell types (Figs. 6, 11, and 14). Metoclopramide is known to accumulate in mitochondrial cell fraction after perfusion in the rat lung (Yoshida et al., 1987) and may accordingly alter the morphology of mitochondria in microscopic observations (Figs. 3, 9B, and 13B). The apoptotic response to metoclopramide or declopramide previously documented in other cell types may derive from mitochondrial alteration, as shown by its reversal by Bcl2 overexpression and by the cytochrome *c* release from the mitochondria to the cytosol (Olsson et al., 2002). In addition, metoclopramide reportedly causes DNA strand breaks and inhibits DNA repair in vitro (Olsson et al., 1995), a possible basis for agent-specific toxicity. After mild damage to DNA, PARP activates the repair machinery, but after severe damage, the enzyme activity of PARP may promote necrosis/apoptosis by depleting NAD^+ and ATP and other mechanisms (Jagtap and Szabó, 2005).

We confirm that the inhibition of NF- κ B nuclear translocation has its own structural basis, being shared by the most highly substituted 4-aminobenzamides (NAPA, metoclopramide, and NAMA) but not procainamide (Fig. 7B, Introduction). The inhibitory effect of metoclopramide on NF- κ B signaling is not dependent on V-ATPase (Fig. 7C) and may be dependent on a direct effect of the drug on I κ B kinase isoforms, as described previously (Liberg et al., 1999; Lindgren et al., 2001, 2003). Benzamide analogs have anti-inflammatory properties and reduce the secretion of pro-inflammatory cytokines such as tumor necrosis factor α , interleukin-1 β , interleukin-6, and interleukin-8 (Pero et al., 1999). The study of the expression of the kinin B_1 receptor, a highly regulated process dependent on NF- κ B in rabbit smooth muscle cells (Sabourin et al., 2002), shows that metoclopramide inhibits the process as expected (Fig. 7D). However, procainamide, which exerts no effect on NF- κ B (Fig. 7B), can also reduce the membrane B_1 receptor density at a concentration that promotes cell vacuolization (Fig. 7D). This decrease was partly reversed by bafilomycin A1 cotreatment, unlike that produced by metoclopramide. Similar to α -fetoprotein secretion inhibition in the Morris 7777 cells (Fig. 10), procainamide-induced vacuolization may impair the trafficking of the B_1 receptor in the secretory pathway independently of NF- κ B. This nonspecific antisecretory action may apply to some cytokines in a previously cited study (Pero et al., 1999). NF- κ B activation can also protect cells from oxidative injury (Chen and Cedarbaum, 1997) and induce the transcriptional activation of multiple genes that suppress apoptosis (Baldwin, 2001). The inhibition of NF- κ B is one plausible basis for the observed radiosensitization and cytotoxicity induced by metoclopramide in tumor cells (Olsson et al., 1997; Pero 1999).

A full understanding of the pharmacological and toxicological actions of 4-aminobenzamide is necessary before their eventual use as antineoplastic agents (Olsson et al., 1995,

2002; Villar-Garea et al., 2003). Although some effects of 4-aminobenzamides are independent of V-ATPase-driven ion trapping (I κ B inhibition, agent-specific cytotoxicity, PARP cleavage), other effects are dependent on this phenomenon (vacuolization, inhibition of secretion, a small component of the cytotoxicity, possibly mitotic arrest after the buffering of acidic organelle contents). All amine drugs in this series are almost equally capable of producing massive vacuolization in several cell types, a reversible but not necessarily innocuous response that was apparently overlooked in previous studies.

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