Morphological Evidence for an Apparent Lysosomotropic Activity by Unsaturated Putrescine Analogues

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ABSTRACT

Treatment of cultured L1210 cells with the putrescine analogue, 2,5-diamino-3-hexyne, at 0.5 mM resulted in the rapid (1–2 h) appearance of numerous cytoplasmic vacuoles which were highly visible by light microscopy. Ultrastructural examination revealed that the vacuoles contained numerous membrane vesicles and electron-dense structures resembling endosomal elements. Other cellular organelles were unaffected by the drug. The overall morphological effect by 2,5-diamino-3-hexyne was nearly identical to that produced in the same cells by the known lysosomotropic agent, chloroquine. Since the putrescine analogue, 1,4-diamino-2-butene at 1.2 mM, also produced comparable cytoplasmic vacuolation, and putrescine itself failed to do so at concentrations as high as 5 mM, it was concluded that the apparent lysosomotropic activity of the putrescine analogues was probably due to their weaker basicity related to the presence of an internal triple bond. Although it is uncertain whether the effect of the analogues on the endosomal system is related to the natural function of polyamines, the finding points out the previously unrecognized potential for certain polyamine analogues to act in this manner.

INTRODUCTION

For some time now, polyamine analogues have been proposed and studied as potential anticancer agents [reviewed by Porter and Sufrin (1)]. Interest in this area intensified with the realization that the elevation in polyamine biosynthetic activity, known to be associated with rapid cell growth, is actually a critical component of the process and not simply a consequence of it. For those analogues bearing close structural resemblance to the polyamines themselves, one or more of several mechanisms could occur within the cell. These include: (a) direct inhibition of polyamine biosynthetic enzymes (2–4); (b) negative regulation of one or more of these biosynthetic enzymes (5, 6); (c) competition with natural polyamines for critical binding site(s); and/or (d) substitution for polyamines at binding sites with subsequent interference with macromolecular structure and/or function. Conceivably, any one of these events could lead to inhibition of cell growth. However, because the role of polyamines in cell proliferation has yet to be determined, it is difficult to distinguish analogue effects related to polyamine function (as proposed in c and d) from those which are not.

For analogues which are only marginally similar in structure to polyamines and, in particular, those bearing biologically reactive groups, myriads of additional possible sites of action unrelated to the function of natural polyamines could be considered. This seems to be the case in the present study in which, in the course of our evaluation of certain polyamine analogues provided by the CIBA-GEIGY, Ltd. (Basel, Switzerland), it was found that treatment of cultured cells with the PUT analogue, DAH (Fig. 1), produced rapid and distinctive morphological effects which seemed to correlate with inhibition of cell growth. In the present communication, we describe these effects and relate them to what appears, on the basis of morphology, to be a selective interference with the endosomal system.

MATERIALS AND METHODS

Materials. DAH (CGP 24438A) was synthesized and made available by CIBA-GEIGY. Chloroquine was obtained from Sigma Chemical Co. (St. Louis, MO). 1,4-Diamino-2-butene was generously provided by Dr. Warren Heston (Memorial Sloan Kettering, New York, NY) who originally obtained it from Calbiochem-Behring (San Diego, Ca.). Cell Culture. Murine L1210 leukemia cells were maintained in logarthmic growth in RPMI 1640 medium containing 2% (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-3-((N-morpholino)propanesulfonic acid, 1 mM aminoguanidine, and 10% Nu Serum (Collaborative Research, Inc., Lexington, MA). Cells were grown in either glass culture tubes in a total volume of 2 ml or 25- or 75-cm² tissue culture flasks in a total volume of 15 and 50 ml, respectively, under a humidified 5% CO₂ atmosphere at 37°C. Varying concentrations of PUT or PUT analogue were pipetted into cell suspension while in logarthmic growth (0.5–1.0 x 10⁶ cells). At various times afterward, cells were removed from tubes for counting and viability determinations and for morphological study. An electronic particle counter (Model ZF Coulter counter; Coulter Electronics, Hialeah, FL) was used to determine cell number which was periodically confirmed with hemocytometer measurements. Trypan blue dye exclusion test (0.5% in unbuffered 0.9% NaCl solution) was done to determine cell viability. The percentage of control growth was determined as follows:

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\% \text{ of control growth} = \frac{\text{Final treated cell number} - \text{initial inoculum} \times 100}{\text{Final untreated cell number} - \text{initial inoculum}}
\]

Light and Electron Microscopy. Drug-treated and untreated cell samples (~10⁶/sample) were washed with cold PBS and then fixed for 24 h at 4°C in 0.1 M phosphate buffer (23 ml 0.2 M NaH₂PO₄·H₂O, 77 ml 0.2 M Na₂HPO₄, 100 ml distilled water, and 0.5 ml 1% CaCl₂) containing 3% glutaraldehyde (pH 7.4, 480 mOsml). Pelleted fixed cells were washed overnight in phosphate buffer, postfixed in phosphate-buffered 1% osmium tetroxide at 4°C for 2 h, dehydrated in a graded alcohol series, and embedded in Epon/Araldite plastic resin. Semithin sections (~500 nm) were prepared for light microscopy with a Porter Blum MT-1 ultramicrotome (Sorvall Corp., Norwalk, CT), stained with 1% aqueous toluidine blue containing 1% sodium borate, and examined and photographed with a Leitz-Wetzlar light microscope. Thin sections (~90 nm) were stained sequentially with uranyl acetate and lead citrate and then examined with a Siemens Elmiscop 101 electron microscope at 80 kV.

Polyamine Pool Determinations. L1210 cell samples were washed twice in cold PBS, and an aliquot of 10⁶ cells was removed for polyamine determination. The cells were pelleted, and the PBS supernatant was carefully removed with a cotton swab. The remaining cell pellet

* The abbreviations used are: PUT, putrescine; DAH, 2,5-diamino-3-hexyne or 1,4-butyne-2-diamine; IC₅₀, 50% growth inhibitory concentration; PBS, phosphate-buffered saline.
lysosomal nature of the vacuoles appeared similar to a generalized swelling of organelles such as is associated with the vacuole, giving the overall appearance of a "signet ring" (Fig. 1). The latter suggested involvement of the endosomal system. In early morphological effects were characterized by electron microscopy. By 2 h, many of the vacuoles were filled with numerous membranous vesicles and electron-dense structures (Fig. 3). Interestingly, other cellular organelles including the endoplasmic reticulum, Golgi apparatus, and mitochondria were unaffected by drug treatment, indicating that DAH is selective in its morphological effects and that it does not induce a nonspecific cytotoxic response.

Interestingly, a PUT analogue, 1,4-diamino-2-butynyl, which closely resembles DAH but lacks the methyl groups, also produced extensive cytoplasmic vacuolation. By electron microscopy, the lysosomal nature of the vacuoles appeared similar to that produced by DAH but required slightly higher concentrations to achieve a comparable effect (Fig. 4). At 0.5 mM, both growth inhibition and cellular vacuolation were minimal with 1,4-diamino-2-butynyl, but at 1.2 mM (the approximate IC₅₀ concentration), both effects approached those produced by 0.5 mM DAH [i.e., 57% growth inhibition and extensive vacuolation (Fig. 4)]. Treatment of cells with concentrations of PUT up to 5 mM did not significantly inhibit cell growth and failed to induce similar cytoplasmic vacuolation.

As a further indication of the morphological origin of the vacuoles, cells were treated with the well-known lysosomotropic agent, chloroquine (7–11), and compared morphologically with cells treated with DAH. While less impressive with respect to vacuole size and number (Fig. 5a), the morphology of the vacuoles induced by 50 μM chloroquine was clearly similar to that produced by DAH and the analogue, 1,4-diamino-2-butyne. After 3 h, vacuoles were observed which contained numerous membrane vesicles and electron-dense structures (Fig. 5b). As with cells treated with DAH, other cellular organelles were not affected by chloroquine.

Finally, we attempted enzyme cytochemistry by staining for acid phosphatase to demonstrate that the vacuoles were part of the lysosomal system. Control and treated whole L1210 cells were smeared onto slides, fixed for 5 min with cold acetone, and stained for acid phosphatase using the naphthol-AS-BI-phosphate fast red violet coupling method (12). Red staining indicative of enzyme product appeared in control cells as punctate bodies clustered to one side of the nucleus. Substrate-deleted controls did not stain for enzyme activity. Contrary to expectations, there was no increase in acid phosphatase staining during treatment with 1 mM DAH for up to 16 h or with 50 μM chloroquine for up to 6 h. Owing to the extremely small size of the stained organelles and difficulty in observing them in the plane of focus of the cell, the preparation could not be effectively documented by photography.

The findings are not necessarily inconsistent with the existence of a lysosomotropic effect as previously described (8) since the increase in staining could be cell type dependent. Failure of chloroquine to enhance staining reinforces this possibility. Thus, macrophages (8), but not L1210 cells, may show an increase in enzyme staining during short treatment conditions because they are biologically equipped for rapid lysosomal enzyme synthesis. Alternatively, the effect could involve nonlysosomal elements of the endosomal system which may possess drug-trapping capabilities but lack acid phosphatase activity.

**DISCUSSION**

On the basis of morphological evidence alone, it seems apparent that the biological effect of the PUT analogue, DAH, is directed at lysosomal structure and function. In fact, on the basis of mechanistic considerations (discussed below), it is possible that the effect could include elements of the entire endosomal pathway. At their early stages of development, cytoplasmic vacuoles induced by DAH contain numerous ele-

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**RESULTS**

It was determined that, over the course of a 48-h incubation, the IC₅₀ for DAH was 0.5 mM, while that for PUT itself was greater than 5 mM. In both cases, the determinations were performed in the presence of the serum diamine oxidase inhibitor, aminoguanidine and the semidefined serum substitute, Nu Serum (Collaborative Research, Inc.) in order to minimize the toxic contribution of enzyme oxidation products. Likewise, interference with polyamine biosynthesis was probably not involved in growth inhibition, since the polyamine pools of growth-inhibited cells were largely unperturbed by DAH for up to 48 h (Table 1).

In cells treated with IC₅₀ doses of DAH, distinctive morphological effects were observed by light microscopy to occur very rapidly (Fig. 2). By 2 h, cells contained numerous vacuoles of similar size distributed throughout their cytoplasm. It is important to note the uniformity of the effect in that all cells seemed to be comparably affected. These vacuoles fused with one another and increased in size as the incubation continued until 12 h. To gain an indication of the reversibility of the cytoplasmic vacuolation, cells were treated for 12 h with 0.5 mM DAH and then placed in drug-free media. By 24 h, the vacuolation was completely resolved (Fig. 2f) indicating that the effect was reversible.

In an attempt to determine the origin of the vacuoles, the early morphological effects were characterized by electron microscopy. By 2 h, many of the vacuoles were filled with numerous membranous vesicles and electron-dense structures (Fig. 3). The latter suggested involvement of the endosomal system. In many instances the vacuoles seemed to fuse with one another so that several cells contained a single larger membrane-bound vacuole, giving the overall appearance of a "signet ring" (Fig. 3). Interestingly, other cellular organelles including the endoplasmic reticulum, Golgi apparatus, and mitochondria were unaffected by drug treatment, indicating that DAH is selective in its morphological effects and that it does not induce a generalized swelling of organelles such as is associated with nonspecific cytotoxic responses.

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Fig. 2. Light micrographs of toluidine blue-stained Epon thick sections of control L1210 leukemia cells (a) and cells treated in vitro with 0.5 mM DAH drug for 2 h (b), 6 h (c), 9 h (d), 12 h (e), and cells treated for 12 h and then placed in drug-free media for 24 h (f). Note that all cells seem to be uniformly affected (b–e) and that the vacuoles become inconspicuous when treated cells are placed in drug-free media (f). × 900.

Iron-dense structures typical of the lysosomes as well as small uniform membrane vesicles, possibly endosomes, which presumably accumulate as a result of interference with membrane-cycling mechanisms (13). The selectivity of this effect was evident by the lack of obvious structural damage to other cellular organelles including the endoplasmic reticulum and mitochondria. The latter is in contradistinction to the effects of another polyamine analogue and inhibitor of biosynthesis,
methylglyoxal-bis(guanylhydrazone). This spermidine analogue specifically affects the structure (14, 15) and function (16) of mitochondria without altering other cellular structures including those related to the endosomal system.

The probability of an endosomal effect by DAH is further reinforced by comparative morphological studies with chloroquine, a well-recognized and potent lysosomotropic agent (7–11). The cytoplasmic vacuolation produced by 50 µM chloroquine in L1210 cells was very similar in appearance to that evoked by DAH (Figs. 3 and 4). DeDuve et al. (10) proposed that certain substances can be selectively accumulated in lysosomes by several mechanisms, one of which suggests drug-induced alteration of the intralysosomal milieu. Accordingly, weakly basic substances, such as DAH, may become trapped as a consequence of protonation related to organelle acidity. When the concentration of the base becomes sufficiently high, the intralysosomal pH increases (17) and the organelle swells osmotically to form large vacuoles (9, 18). Endocytic elements and those involved in membrane recycling would be expected to be similarly affected (13) since they have also recently been...
shown to be acidic in content (19) and therefore ultimately able to contribute to the ultrastructural picture. Ohkuma and Poole (11) have compared the activity of a number of weak bases to induce vacuolation in macrophages by the above mechanisms. On a concentration basis, chloroquine was found to be most effective (30 \( \mu \)M) and PUT very ineffective (<5 mM).

It is interesting that DAH elicits the vacuolar response while PUT does not. Because DAH is considerably less basic than PUT (pK 1 and 2: 8.9 and 7.4 versus 10.6 and 9.5, respectively), shifts in molecular charge associated with the presence of an internal triple bond may play a role. Thus, by being less basic than PUT, DAH may be able to cross the lysosomal membrane as an uncharged species. However, once in contact with the low pH of the organelle interior, DAH could become protonated and hence unable to exit leading to an accumulation of the drug (17). Further indication for the role of the triple bond derives from the finding that 1,4-diamino-2-butyne also causes cytoplasmic vacuolation. The greater effectiveness of DAH in this activity may be due to the methyl groups which undoubtedly contribute to its lipophilicity and to its potential for crossing membranes. The analogue, 1,4-diamino-2-butyne, has been used previously by Heston et al. (20) to characterize polyamine uptake into rat prostate-derived tumor cells and, more recently, by Sarhan et al. (21) to characterize the specificity of the spermidine synthase reaction. Whether, by virtue of having an internal double bond, 2,5-diamino-3-hexene might also produce a lysosomotropic effect was not determined.

Given the greater potential of DAH over PUT for reactivity with biological structures, it seems likely that the observed effects are unrelated to those normally mediated by PUT especially since, as noted above, neither PUT nor the more basic polyamines (22), spermidine and spermine, exhibited a similar morphological response (11). The polyamines have, however, been recently implicated in lysosomal function through their ability to stimulate lysosomal transport of cystine, a process essential for the cellular recycling of free cystine obtained by lysosomal proteolysis (23). One significance of the present finding resides in recognition of the fact that apparent lysosomotropic activity remains a potential and significant effect of certain putrescine analogues. It seems probable that similar analogues of higher polyamines, spermidine and spermine, would be unlikely to possess this activity since, by virtue of additional nitrogens, they should retain sufficient basicity to resist membrane transport into the lysosome.

DAH was originally synthesized as a potential antitumor agent and was found to have modest activity against Ehrlich ascites carcinoma \textit{in vivo}.\footnote{J. Janne, University of Helsinki, Helsinki, Finland, personal communication.} The early and profound effects of DAH on the endosomal system of cultured cells at concentrations which are growth limiting suggest that the two events might be related. Indeed, concentrations of DAH which are not growth inhibitory were not found to have apparent lysosomotropic effects. Similarly, reversal of DAH-induced vacuolation in drug-free media was accompanied by a resumption in cell growth. It is uncertain to what extent the lysosomotropic activity of chloroquine contributes to its profound antimalarial activity (24–26). It has been proposed (26) that in red blood cells, chloroquine might interfere with the lysosomal digestion of hemoglobin by the parasite. If this were true, DAH might also be expected to exhibit antimalarial activity. Similarly, it may be effective as an antiinflammatory agent.

ACKNOWLEDGMENTS

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Fig. 5. L1210 cells treated for 3 h with 50 μM chloroquine. At low magnification (a), numerous vacuoles are present in the cytoplasm, while other cellular organelles appear normal. Under higher magnification (b), the vacuoles are seen to contain numerous electron-dense structures and membrane vesicles (arrow) similar to those seen in cells treated with DAH (Fig. 3b). a. × 8,200; b. × 20,000.

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