Brief Communication

Differences in Cellular Uptake and Cytofluorescence of Adriamycin and N-Trifluoroacetyladriamycin-14-valerate

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SUMMARY

Adriamycin-specific fluorescence appears slowly in living cells and is localized in nuclei and chromosomes. N-Trifluoroacetyladriamycin-14-valerate, a recently synthesized adriamycin analog, differs from the parent anthracycline in the rapid appearance of its fluorescence in the cytoplasm of living cells and the lack of any fluorescent binding to nuclei and chromosomes.

INTRODUCTION

Adriamycin and daunorubicin are anthracycline antibiotics with significant antitumor activity in both experimental tumors and in human cancers (2, 13). Intercalation of daunorubicin and adriamycin between adjacent base pairs of the DNA double helix, and the resulting loss of template activity, has been proposed as the mechanism for the antitumor action of these antibiotics (1, 2). Consistent with this hypothesis are observations on the binding of these drugs to various DNA preparations, their effect on DNA and RNA polymerase-mediated reactions, and their inhibitory effect on labeled precursor incorporation into nuclei acids (1, 2, 6, 9, 12, 13). The appearance of drug-specific fluorescence in nuclei and in chromosome bands of cells exposed to adriamycin or daunorubicin further points to the DNA binding affinity of these drugs (3, 4, 8, 12).

AD 32 (NSC 246131), an adriamycin analog recently synthesized and currently being investigated here, is therapeutically superior to and less toxic than adriamycin in a number of experimental rodent tumor systems (5, 10). In connection with studies on the mechanism of action and the cytokinetic effects of adriamycin and its analogs (7), we have found striking differences between adriamycin and AD 32 in the rate of appearance and localization of drug-specific fluorescence. This report describes these preliminary observations.

MATERIALS AND METHODS

Log-phase suspension cultures (1 to 2 × 10⁶ cells/ml) of CCRF-CEM human lymphoblasts were grown in Eagle's minimal essential medium (for spinner cultures). Human fibroblasts of the WI-38 cell line were grown as monolayer on coverslips in Leighton tubes and nourished with Eagle’s minimal essential medium. Tissue culture media were supplemented with 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). CCRF-CEM cells (10 ml) from stock cultures (1 × 10⁶ cells/ml) were centrifuged at 500 × g for 5 min and resuspended in 1 ml of fresh drug-containing medium at 4° or 37°. WI-38 cells growing on coverslips were incubated in fresh drug-containing medium. Stock solutions (1 mg/ml) of adriamycin hydrochloride and AD 32 were made in 10% Tween 80.

Serial dilutions (1 to 10 μg/ml) were made in Hanks’ balanced salt solution before addition to the cultures. Cells were examined and photographed under a Zeiss phase contrast-UV fluorescent microscope equipped with a high-pressure mercury light source. Excitation and barrier filters were BG 12 (peak transmission, ~350 to 450 nm) and Zeiss 53 (transmission, >525 nm), respectively. Photomicrographs were taken on Kodak Tri-X film.

RESULTS

Adriamycin. In CCRF-CEM lymphoblasts and WI-38 cells (live) incubated with adriamycin (1 to 10 μg/ml) for 30 min at 37°, no intracellular (cytoplasmic or nuclear) fluorescence could be visibly detected. After incubation for 3 hr, very faint intracellular fluorescence could be seen, whereas after 24 hr of incubation, bright pink fluorescence was seen in the nuclei and chromosomes of live cells. In dead cells (with cell membrane function lost and pyknotic nuclei) or in cells fixed in methanol:acetic acid (3:1) for 5 min and incubated with adriamycin (1 min) (1 to 10 μg/ml), bright pink fluorescence was seen in nuclei (Fig. 1a) and chromosomes (Fig. 1b).

AD 32. In contrast to the slow appearance of adriamycin-specific cytofluorescence in live cells, bright pink fluorescence appeared rapidly in the cytoplasm of live cells incubated with AD 32 (1 to 10 μg/ml) for 1 min. No effect of incubation temperature (4 or 37°) or length of exposure (1 min to 24 hr) was seen on either the appearance or localization of AD 32 fluorescence. Fig. 1, c and d, shows CCRF-CEM and WI-38 cells, respectively, incubated with AD 32 (1 μg/ml) at 4° for 5 min; brilliant cytofluorescence can be seen in the cytoplasm but not in the nuclei. In cells fixed with methanol:acetic acid (3:1) for 5 min prior to incubation.
with AD 32 (1 to 10 μg/ml), a similar localization of drug-specific fluorescence in cytoplasm, but not in the nuclei, was seen. No nuclear fluorescence was detectable in cells incubated with higher concentrations of AD 32 (50 μg/ml) or exposed to the drug for up to 24 hr. regardless of whether the cells were live or fixed in methanol:acetic acid. Similarly, and in contrast to adriamycin, no fluorescent staining of chromosomes was seen in prefixed (methanol:acetic acid. 3:1, for 5 min) preparations stained with AD 32 (1 to 100 μg/ml). Fig. 1, e and f, shows, respectively, a phase-contrast and a UV-fluorescent microscope picture of a live WI-38 cell incubated with AD 32 (1 μg/ml) for 5 min. A comparison of these 2 micrographs shows that AD 32-specific cytoplasmic fluorescence (Fig. 1f) is associated with cytoplasmic structures that appear as dark spheres under phase contrast (Fig. 1e).

**DISCUSSION**

AD 32 is superior to adriamycin in the chemotherapy of mice bearing the L1210 and P388 leukemias (5). In comparable experiments the optimal (most effective nontoxic) dose of AD 32 is 8 to 10 times that of adriamycin on a molar basis. Against the L1210 leukemia in vivo, AD 32 at 50 to 60 mg/kg/day for 4 days reproducibly gave a median increase in life-span of treated animals versus untreated controls greater than +445% with a high percentage of long-term survivors, compared to a median increase in life-span of about +50% and no long-term survivors for adriamycin (4 mg/kg/day for 4 days).

The present study shows that, besides its superior chemotherapeutic efficacy, AD 32 differs from adriamycin in the rapid intracellular appearance and cytoplasmic localization of its cytofluorescence. In contrast to adriamycin, which enters cells slowly by a temperature-dependent transport mechanism (9), and the slow intracellular appearance of adriamycin cytofluorescence, AD 32-specific cytofluorescence appears rapidly in cells incubated at either 4° or 37°. It seems reasonable that the rapid appearance of AD 32 cytofluorescence is related to the much greater lipophilic character of this analog as compared to the parent anthracycline. Our preliminary results from a radioimmunoassay study comparing intracellular transport of adriamycin and AD 32 support the view that the rapid appearance of AD 32 cytofluorescence is related to its rapid transport (or diffusion) across the cell membrane, in contrast to the slower, temperature-dependent transport of adriamycin (A. Krishan and V. Raso, unpublished observation).

In addition to the rapid cellular entry of AD 32, the drug-specific cytofluorescence that it exhibits differs from that of adriamycin (3, 4, 8, 12) in its lack of association with nuclei or chromosomes. As shown in the present study, AD 32-specific cytofluorescence is apparently associated with spherical structures in the cytoplasm of WI-38 cells. That these structures are probably not mitochondria is suggested by the observation that, in WI-38 cells stained with the vital dye, Janus green, mitochondria appear not as spherical bodies but rather as long filamentous structures.

The absence of AD 32 fluorescence in nuclei and chromosomes is also consistent with our preliminary findings on the lack of AD 32 binding to calf thymus DNA (11). These observations are also in keeping with the proposed structural requirements for the binding of anthracyclines to DNA (1, 2). In its structural formula, AD 32 differs from adriamycin in having a 5-carbon straight-chain ester function at the 14-carbinol position and trifluoroacetyl substitution on the glycosidic amino group. According to the currently accepted mechanism for the antitumor activity of daunorubicin and adriamycin, the basic glycosidic amino group must be free for DNA binding and for demonstration of biological activity (1, 2). In AD 32 this position is occupied by the trifluoroacetyl substitution, and this appears to account for its lack of binding to DNA (11). In spite of this, however, AD 32 shows significant biological and chemotherapeutic activity both in vivo and in vitro. It therefore appears that either AD 32 is metabolized to a DNA-binding metabolite or its effect on cells in vivo and in vitro is not a consequence of DNA binding. Further studies are underway to elucidate the mechanism of action of this unique adriamycin analog.

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**REFERENCES**

Fig. 1. a and b, Fluorescent nuclei of dead WI-38 cells (a) and nuclei and chromosomes of methanol:acetic acid-fixed CCRF-CEM cells (b) incubated with adriamycin (1 μg/ml). a, × 720; b, × 600. c and d, CCRF-CEM lymphoblasts (c) and WI-38 fibroblasts (d) exposed live to AD 32 (1 μg/ml). Note the localization of cytofluorescence in cytoplasm and not in the nuclei. × 600. e and f, a phase-contrast (e) and a UV-fluorescent microscope picture (f) of a live WI-38 fibroblast exposed to AD 32 (1 μg/ml). × 1500.
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