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NOVEL MODE OF CYTOTOXICITY OBTAINED BY COUPLING INACTIVE ANTHRACYCLINE TO A POLYMER*

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Abstract—The inactive anthracycline analog 4-demethoxy-7,9-di-epi-daunorubicin was covalently coupled to polyglutaraldehyde microspheres. The polymer-bound analog acquired significant cytostatic activity as evaluated with doxorubicin resistant and sensitive murine L1210 leukemia cells. A suggested multiple membrane interaction at the cell surface may represent a novel mechanism of cytotoxicity.

Since the inception of the widely used chemotherapeutic agent adriamycin (Adr), DNA has been cited as the primary drug target [1, 2]. The DNA receptor hypothesis finds its justification from the fact that the drug binds to double-stranded DNA with high affinity [3] and appears to accumulate in the nuclei of treated cells [4]. Cytotoxic effects are, therefore, thought to be the direct result of inhibition of DNA or RNA synthesis through strand damage or breakage [5–7]. However, there are concerns about the validity of the DNA-receptor theory. For instance, no obvious correlation exists between cytotoxicity and number of strand breaks [8] or between cell viability and inhibition of DNA or RNA synthesis [9]. In addition, *N*-trifluoroacetyl adriamycin-14-valerate is an Adr analog which does not bind to DNA but has greater cytotoxic activity than Adr, suggesting that DNA binding is not mandatory for anthracycline activity.

The cell surface was observed to be a potential site of Adr action based on observations that anthracyclines are known to modulate many membrane-related activities including glycoprotein synthesis [10], expression of growth hormone receptors [11], fluidity properties [12], and microtubule assembly [13]. Evidence that Adr cytotoxicity could actually be mediated through a membrane-dependent mechanism came from studies in which the drug was covalently linked to a polymer solid phase support which could not enter cells [14–18, ‡]. Results demonstrated that, when Adr interactions were limited to the cell surface, the cytotoxic characteristics of the drug were retained as well as amplified. It was proposed that the increase in activity was due to multiple interactions of the polymer-bound drug with components of the plasma membrane, resulting in an inescapable perturbation at the cell surface [14, ‡]. In addition, it was demonstrated with various cell lines that the polymer-bound drug could overcome resistance to free Adr. Multiple binding and continuous perturbation by polymer-bound drug could overcome any of the three major resistance mechanisms thought to occur for free drug. For example, bound drug would not be subject to (a) increased intracellular enzymatic degradation, (b) decreased uptake, or (c) increased efflux [16, 17, ‡].

Observations made with the polyglutaraldehyde microspheres and with the agarose bead-bound drug raised the question of whether the original mode of Adr cytotoxicity has been simply amplified or if drug attachment actually created a new mechanism of cell killing. To answer this question, we have chosen an inactive Adr analog, 4-demethoxy-7,9-di-epi-daunorubicin (7R-9R), and covalently coupled it to polyglutaraldehyde microspheres. The rationale was that, if the same mechanism of drug action prevailed for the two forms of Adr, the polymer-bound analog would remain inactive. Here we report the newly acquired cytostatic activity of this analog.

METHODS AND MATERIALS

Chemicals. Fetal calf serum (FCS), fungizone, and penicillin–streptomycin were obtained from Flow Laboratories, McLean, VA. The 4-demethoxy-7,9-di-epi-daunorubicin was the gift of Dr. A. M. Casazza and Dr. Penco, Farmitalia Research Laboratories, Milano, Italy. Sodium hydroxide and sodium chloride were purchased from the Sigma Chemical Co., St. Louis, MO. RPMI-1640 was obtained from GIBCO, Grand, Island, NY. The detergents Aerosol 604 and Nonidet P-40 (NP-40) were obtained from American Cyanamide, Wayne, NJ, and Particle Data Laboratories, Elmhurst, IL, respectively.

Synthesis of microspheres. A solution of 10% glutaraldehyde and 0.1% Aerosol 604 at pH 11.0 was reacted at room temperature with stirring for 24 hr [16]. The pH of the solution was readjusted every 60 min with 20% NaOH during the initial 6 hr. The resulting polyglutaraldehyde microspheres (PGLs) were washed exhaustively with sterile distilled water. The yield for the synthesis of microspheres was determined by drying aliquots under vacuum at 40°.

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‡ K. E. Rogers and Z. A. Tökés, unpublished observations.

Coupling of drug. The analog 7R-9R was dissolved in sterile distilled water to give a final concentration of 2.5 mg/ml. Thereafter, the solution was kept at 4° in darkness. The drug coupling was initiated by adding 5 mg of 7R-9R to 100 mg of PGLs at pH 6.0. PGLs and 7R-9R analog were stirred together for 4 hr at room temperature in darkness. To stop the reaction the microspheres were centrifuged at 10,000 *g* for 30 min. The supernatant fraction was retained, and the microspheres were washed five times with 20 ml of sterile distilled water. Labeled microspheres were then treated with 10 ml of 0.5% NP-40 for 2 hr. Treatment was followed by exhaustive washing with water until no additional 7R-9R could be removed from the microspheres, as determined by optical absorption. Since a radioisotope-labeled 7R-9R analog was not available, the amount of drug bound to the microspheres was calculated by determining the concentration of free 7R-9R remaining in the washes using spectrophotofluorescence and subtracting it from the total amount of drug originally added. The values obtained were confirmed spectrophotometrically. The accuracy of determining the amount of bound drug ranged from 3 to 6%.

Uncoupled microspheres for controls (P-PGLs) were obtained by reducing the aldehyde groups of 100 mg of plain microspheres with 0.1% KBH_4 for 1.5 hr. The reduced microspheres were washed extensively with sterile water to remove unreacted KBH_4 and subsequently treated with 10 ml of 0.5% NP-40 for 2 hr. Detergent was removed from the microspheres by exhaustive washing.

Cytostatic assays. L1210 sensitive and resistant cell lines ($L1210_S$ and $L1210_R$ respectively) were maintained in suspension culture in RPMI-1640 medium supplemented with 10% FCS, 0.5 mg/ml penicillin–streptomycin, and 0.015 mg/ml fungizone. $L1210_R$ cells were obtained by growing $L1210_S$ cells (Dr. T. Moore, Roswell Park, NY) in the presence of 10^{-10} M Adr for 4 weeks followed by a shift to 10^{-9} M Adr for an additional 8–10 weeks. In all experiments, $L1210_R$ cells were grown in non-selective medium for 2–3 weeks before they were used.

Growth inhibition samples were initiated by diluting cells to 15,000 cells per assay per 1.8 ml medium and aliquoting 1.8 ml of the suspension into sterile 12 × 75 mm test tubes. Free or bound 7R-9R was dispersed in isotonic saline, and a 200-μl aliquot of the appropriate concentration was added to each tube. The samples were covered and incubated at 37° in the presence of 5% CO_2 for four to six doubling times. At the end of incubation, the cells were gently resuspended by votexing, and the final cell number was determined on a model B Coulter Counter.

RESULTS

Glutaraldehyde polymerization produced microspheres ranging in size from 2000 to 6500 Å [16]. There were approximately 2×10^{12} microspheres present in 100 mg of polymer, and 1.52 mg of 7R-9R was coupled to this number of PGLs. This represents a coupling efficiency of 1.52% and therefore implies that 8.3×10^5 molecules of 7R-9R were attached per microsphere. No detectable drug was released from microspheres dispersed in saline for periods exceeding 4 weeks as determined by spectrophotofluorescence. Stability of the covalent bonds between the analog and the microspheres during incubation with cells was indistinguishable from the previously reported data with Adr [16].

The growth inhibition properties of 7R-9R and 7R-9R-PGLs with $L1210_S$ are shown in Fig. 1. Free drug had no effect on growth at concentrations up to 10^{-6} M; however, the bound drug inhibited more than 95% of the growth at this concentration. The concentration at which 50% of the growth was inhibited (IC_{50}) was determined to be 2.1×10^{-7} M for the 7R-9R-PGLs and virtually no growth was observed at 10^{-5} M. The identical number of P-PGLs had no

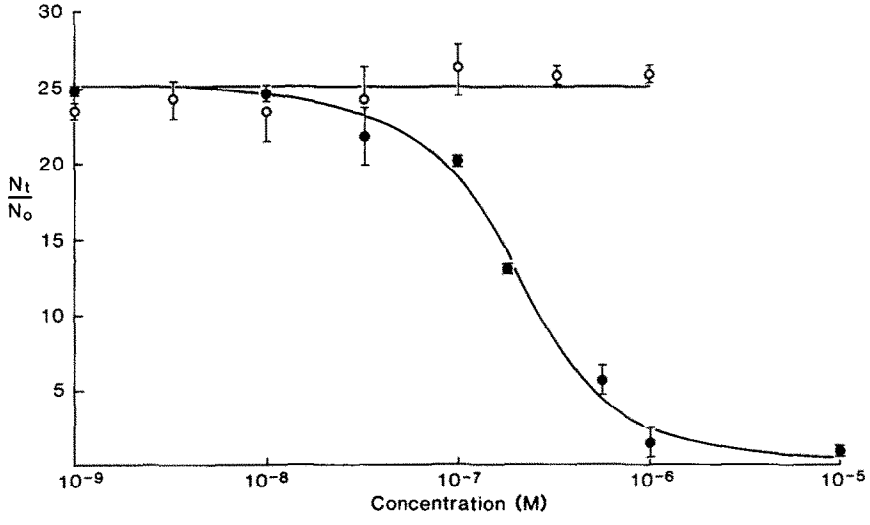

Fig. 1. Comparison of growth inhibition effects of free 7R-9R and coupled 7R-9R using $L1210_S$ leukemia cells. N_t is the total number of cells present after four to six doubling times, and N_o is the inoculum density. Concentration is the molarity of either free or bound 7R-9R. Each point represents the mean of at least four determinations. Key: Free 7R-9R (○—○) and 7R-9R-PGLs (●—●). Bars represent the S.E.

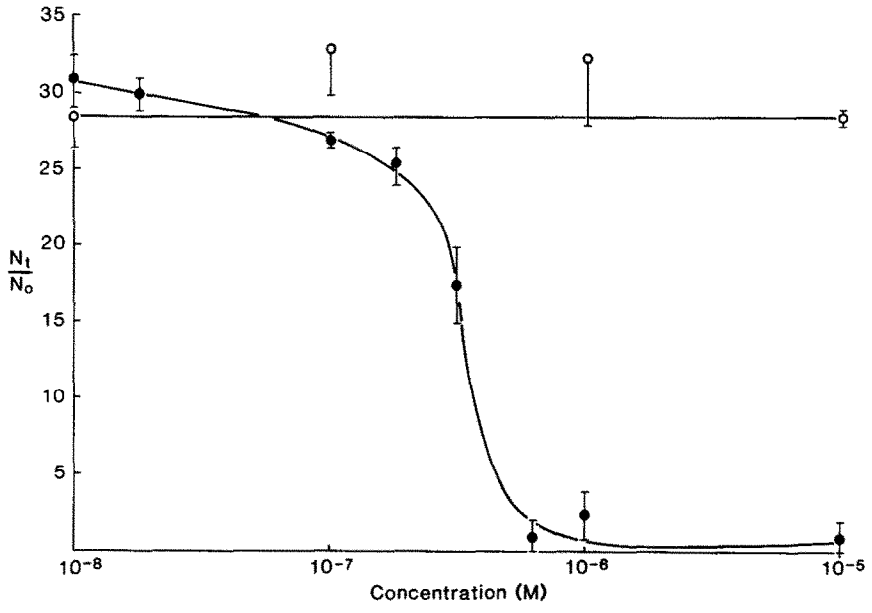

Fig. 2. Comparison of growth inhibition effects of free 7R-9R and coupled 7R-9R using $L1210_R$ leukemia cells. N_t is the total number of cells present after four to six doubling times, and N_o is the inoculum density. Concentration is the molarity of the free or bound drug. Each point is the mean of at least four determinations. Key: free 7R-9R (O—O) and 7R-9R-PGLs (●—●). Bars represent the S.E.

detectable effect on the $L1210_S$ cells at the concentration equivalent to 10^{-6} M drug; this translates into approximately 3000 plain microspheres per cell. Conversely, it required less than 10 microspheres carrying the inactive analog to begin to exert growth inhibition of the $L1210_S$ cells. Approximately 600 microspheres per cell were present at the IC_{50} concentration of 7R-9R-PGLs after incubation for four to five doubling times.

Growth inhibition studies for the free and bound 7R-9R with $L1210_R$ cells are depicted in Fig. 2. Free 7R-9R had no effect on growth even at the highest concentrations of 10^{-5} M, while the bound drug had demonstrable effects at 10^{-7} M. The IC_{50} for the bound drug was determined to be 3.2×10^{-7} M. Again, P-PGLs equivalent in number to bind 10^{-6} M 7R-9R had no effect on cell growth. An additional control experiment was included in which enough P-PGLs to bind 10^{-6} M 7R-9R were tested together with 10^{-9} M free 7R-9R for growth inhibition. The concentration of free drug was chosen to reflect a minimum of a 10-fold increase in the amount of drug which was known to be released from the Adr-PGLs [16]. This particular control was included to detect any synergistic inhibition effects which might result from the presence of a small amount of free drug together with a large number of microspheres. The combination of free drug and microspheres did not cause growth inhibition with either the $L1210_S$ or $L1210_R$ cell line. The observation suggests that even very large numbers of plain microspheres do not perturb the cells to the extent that a small amount of free drug would become cytotoxic.

* K. E. Rogers and Z. A. Tökés, unpublished observations.

DISCUSSION

The 7R-9R analog of daunorubicin differs from the parent compound at position 4 where a methyl group has been deleted, and also bears an inverted configuration in positions 7 and 9. The compound is not cytotoxic [19] and does not have a significant *in vivo* antitumour activity [20]. Lack of 7R-9R cytotoxicity was easily reconfirmed from the growth inhibition curves presented in this study, whereby free drug concentrations as high as 10^{-5} M had no effects. The lack of activity is thought to be a direct result of the fact that 7R-9R does not bind to DNA and shows a reduced inhibition of DNA synthesis as well as a reduced effect on nucleic acid polymerases [21]. It has been demonstrated that decreased 7R-9R activity is not related to cellular uptake, since influx of this analog is more rapid and reaches higher levels than either daunorubicin or adriamycin [19, 22]. However, the exact intracellular distribution of this analog is not known.

The results from Figs. 1 and 2 demonstrate that covalently coupling the nonactive analog to a solid support caused the drug to become cytostatic. This activity was not due to P-PGLs since they have no effect on growth. Furthermore, since the combination of P-PGLs and free 7R-9R did not affect growth, it is unlikely that the P-PGLs perturb the cells enough to make the drug active.

Detailed analyses of endocytosis of anthracycline-coupled microspheres by hepatocytes and L1210 cells using transmission electronmicroscopy were completed recently [17, *]. Evaluation of the position of 3000 microspheres in relationship to 70 murine leukemia cells revealed that approximately 20% of the microspheres were in close contact with the cell

surface after 12-hr incubations at a drug-equivalent concentration of 10^{-7} M. Less than 0.5% of the microspheres were located intracellularly.* At an IC_{50} concentration of 7R-9R, fewer than 3 microspheres per cell were endocytosed. The covalently bound anthracyclines remain attached to the microspheres during the cytostatic assay. Less than 0.08% of the drug is released during incubation with the cells [17]. Therefore, the estimated number of anthracycline molecules per cell is 2000 or less, which amounts to a cellular concentration of 3.4×10^{-10} M. This is three thousand times less than the necessary amount of free analog to produce any cytostatic activity on the sensitive L1210 cells (Fig. 1). Based on these estimations, it is proposed that the cytotoxic activity of 7R-9R-PGLs could be the result of interactions between the bound analog and the cell surface [16, 17, *]. It is possible that these surface interactions are directly related to the lipophilic nature of the analog. For instance, 7R-9R shows a relatively high affinity for lipids which is substantiated by its 1-butanol/buffer partition coefficient and from its marked uptake by cells [19]. The exact molecular configurations of the analog at the microsphere surface are not yet defined. It is, however, reasonable to postulate that the drug bound to a microsphere would still retain affinity for lipids and would therefore interact with components of the membrane bilayer. Because an estimated 833,000 molecules of 7R-9R are bound to each microsphere in close proximity to one another, the contact of a microsphere with a cell surface will result in several thousand interactions of the drug with the cell membrane. Indeed, transmission electronmicroscopy revealed an interaction between the plasma membranes and Adr-PGLs which extended over a region of several hundred angstroms.* The consequences of these interactions could be classified into two kinds of events. The 7R-9R interactions could specifically inhibit any one of the processes necessary for viability, such as the Na^+–K^+-dependent ATPase.† Alternatively, the drug may exert its lethal effects less specifically by overwhelming and continuously perturbing several membrane associated functions simultaneously. Such amplified and prolonged perturbations would be negligible or non-existent for the free drug. Since the free drug is highly lipophilic and rapidly diffuses into the cell, membrane repair mechanisms should be able to compensate for the small amount of damage incurred. Such a repair mechanism may not be available or adequate against the solid-phase supported drug. In addition, it is essential to emphasize that those cellular compartments which are readily accessible to the free drug may not be available to the polymer-bound drug. Thus, the polymer-bound drug system is not appropriate to extrapolate to the mode of action for the free drug.

In summary, the study demonstrates that presentation of inactive anthracyclines on a solid-phase support resulted in the acquisition of cytotoxic activity. The experiments also suggest that the covalent attachment to polymers of a large variety of amphipathic agents may also result in a desirable cytotoxic activity. In addition, our observations call for a caution in interpreting the results from previous investigations using solid-phase supported Adr and concluding that free anthracyclines can be cytotoxic without entering cells [18]. A more likely interpretation is the creation of a new mechanism of cytotoxicity due to covalent drug attachment to polymers.

* K. E. Rogers and Z. A. Tökés, unpublished observations.

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