# MECHANISM OF ACTION OF THE ANTHRACYCLINE ANTI-TUMOR ANTIBIOTICS, DOXORUBICIN, DAUNOMYCIN AND RUBIDAZONE: PREFERENTIAL INHIBITION OF DNA POLYMERASE $\alpha$*

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The effects of the anthracycline anti-tumor antibiotics, doxorubicin, daunomycin and rubidazone upon $^3$H-TTP incorporating activities of partially purified DNA polymerases and isolated liver nuclei were studied. Doxorubicin, daunomycin and rubidazone inhibited DNA polymerases $\alpha$ and $\beta$ in proportion to drug dose, with consistent preferential inhibition of the $\alpha$ polymerase in comparison to the $\beta$ polymerase for all three drugs. Studies with isolated nuclei, including normal liver nuclei (predominantly $\beta$ polymerase activity), regenerating liver nuclei ($\alpha > \beta$ activity) and Brij 58, $MgCl_2$-extracted regenerating liver nuclei (predominantly $\alpha$ activity), showed inhibition in the order Brij 58, $MgCl_2$-extracted regenerating > regenerating > normal nuclei, corroborating the results obtained with the separate polymerase activities. The elevations in DNA melting temperatures caused by the binding of doxorubicin, daunomycin and rubidazone correlated with the degrees of inhibition of the polymerase activities, suggesting that intercalative binding is the mechanism by which these three agents inhibit the DNA polymerases.

It is suggested that preferential inhibition of the $\alpha$ polymerase (the putative replicative polymerase) in comparison to the $\beta$ polymerase (the presumed repair polymerase) may underlie the Cell Cycle Specific character of the mechanism of action of these anthracycline antibiotics.

The anthracycline antibiotics and their derivatives[1,2] are an important group of anti-tumor agents of which several are clinically useful. Doxorubicin (adriamycin, NSC-123127) is widely used in the treatment of metastatic breast cancer, lymphomas and sarcomas, as well as other human neoplasms[3]. Daunomycin (daunorubicin, NSC-82151), is mainly used in the treatment of acute leukemias[4,5]. Rubidazone (benzoylhydrazone daunorubicine, NSC-164011) has been used extensively in Europe for the treatment of acute leukemias and sarcomas[6], and is currently undergoing further clinical trials in the United States in order to determine whether it offers any advantage over doxorubicin or daunomycin.

The biological activity of these anti-tumor antibiotics is apparently a consequence of complex formation with DNA[7,8]. Doxorubicin and daunomycin avidly bind to DNA by intercalation[9,10], causing an increase in the melting temperature of the DNA[7,11]. The resulting inhibition of DNA synthesis is thought to be secondary to a decrease in the ability of the two strands of the double helix

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of DNA to separate, in preparation for replication, in the presence of intercalated drug[12]. Steric inhibition of DNA polymerase by the bound drug molecule has also been postulated[12]. In addition, several investigators have reported that single strand DNA scissions can be demonstrated both *in vivo* and *in vitro* following exposure to doxorubicin and daunomycin[13~15].

Inhibition of a number of viral and bacterial DNA polymerases by the anthracycline antibiotics has been reported[2,16,17]. However, no studies comparing the effects of these agents upon the presumed replicative and repair polymerases from a single mammalian source have been described.

The purpose of the studies described in this report was to assess the relative effects of the three closely related anthracycline antibiotics, doxorubicin, daunomycin and rubidazone, on DNA polymerase activity, and to determine whether or not these agents produce any differential effect upon DNA polymerase $\alpha$ in comparison to DNA polymerase $\beta$.

## Materials and Methods

### Drugs and Biochemicals

Doxorubicin was generously provided by Adria Laboratories, Inc., Wilmington, Delaware. Daunomycin and rubidazone were provided by Mr. PAUL VILK of the Investigational Drug Branch of the National Cancer Institute, National Institutes of Health. Thymidine 5′-triphosphate (methyl-$^3$H) was a product of New England Nuclear Corp. All other biochemicals were purchased from Sigma Chemical Company.

### Maintenance of Rats and Partial Hepatectomy

Male albino rats (Fischer 344) from Charles River Breeding Laboratories were freely given food and water and used when they weighed between 100 and 150 g. Partial hepatectomy refers to the surgical removal of 2/3 of the liver (left lateral and median lobes)[18]. The regenerating liver remnants were removed 24 hours after the operation.

### Isolation of Liver Nuclei

The isolation of liver nuclei has been described in detail elsewhere[19]. Briefly, the liver samples were homogenized gently in 0.3 M sucrose, 4 mM $CaCl_2$ and filtered through 110 mesh monofilament nylon screen. After a brief, low-speed centrifugation the pellet was resuspended in 2 M sucrose, 1 mM $CaCl_2$, layered over 2.2 M sucrose and pelleted by centrifugation. The nuclear pellets were suspended in 0.3 M sucrose and used immediately or they could be frozen without resuspension in liquid $N_2$ and stored at $-60$°C for at least one month without detectable loss of activity. Extraction of the nuclei with the non-ionic detergent Brij 58 and magnesium was as previously described[20] except that the chloride salt of magnesium was used in place of the acetate.

### Assays of DNA Polymerase Activity

DNA polymerase activity was determined, as previously reported[21], in duplicate reaction mixtures (0.125 ml) containing 0.15 M Tris-HCl pH 8.1, 3 mM $MgCl_2$, 1 mM spermidine, 10 $\mu$g of crystalline bovine serum albumin, $8\times10^{-5}$M each dATP, dGTP, and dCTP, $8\times10^{-6}$M $^3$H-TTP (1 Ci/m mole), 200 $\mu$g of "activated"[22] calf thymus DNA and the DNA polymerase preparation. After incubation at 37°C for 20 minutes the reactions were stopped with 0.5 ml of 1 M NaOH. After the addition of 200 $\mu$g of heat-denatured calf thymus DNA as a co-precipitant, the samples were acidified with 5 ml of 10% trichloroacetic acid, 5 mM $Na_4P_2O_7$, and the insoluble material was collected by centrifugation. This precipitate was then dissolved in 0.5 ml of 1 M NaOH, reprecipitated with trichloroacetic acid, $Na_4P_2O_7$ and centrifuged again. After dissolving the resulting pellet in 0.5 ml of 1 M NaOH and heating at 80°C for 10 minutes, the samples were acidified once again, 75 mg of Celite analytical filter acid was added and the precipitate collected onto pads of 75 mg of Celite. The pads were then sequentially rinsed with trichloroacetic acid, ethanol, and ether and dried. The dry samples were finally heated at 60°C for 10 minutes in 0.5 ml of Hydroxide of Hyamine and counted in 10 ml of a toluene liquid scintillation cocktail containing 4 g of PPO and 50 mg of POPOP per 1 of toluene.

Solubilization and Partial Purification of DNA Polymerase $\beta$

DNA polymerase $\beta$ was extracted from the livers of unoperated rats essentially as described by MEYER and SIMPSON[23]. Nuclei were isolated as described above and suspended in 0.01 M Tris-HCl (pH 8.1), 0.25 M KCl, 5 mM $MgCl_2$, 1 mM EDTA. The nuclear suspension was sonicated and then dissolved by slowly adding 5 M NaCl to a final concentration of 2 M. Nucleohistone was then precipitated by dialysis (overnight) against the same buffer with 0.15 M NaCl. After removal of the precipitate by centrifugation the supernatant was fractionated by treatment with $(NH_4)_2SO_4$ and the protein which precipitated between 40% and 75% saturation was saved. After dialysis against the Tris, KCl, $MgCl_2$, EDTA buffer the preparation was treated with DEAE Bio-Gel. That material which was not adsorbed was concentrated by $(NH_4)_2SO_4$ precipitation and finally dialized against 0.01 M Tris-HCl (pH 8.1) 20% glycerol, 2 mM 2-mercaptoethanol. This activity which sediments at 3.2S in sucrose gradients[21], represented about a sixty-five fold purification over the intact nuclei.

Solubilization and Partial Purification of DNA Polymerase $\alpha$

DNA polymerase $\alpha$ was extracted from the livers of partially hepatectomized rats as previously described[21]. Nuclei were isolated as described above and extracted twice with 5% Brij 58, 50 mM $MgCl_2$, 5 mM 2-mercaptoethanol. The extracted nuclei were then lysed by the addition of 2 M NaCl, 0.01 M Tris-HCl (pH 8.1), 0.5 mM EDTA, and 20 mM 2-mercaptoethanol. The nucleohistone was precipitated by overnight dialysis against 4 mM Tris-HCl (pH 8.1), 5% glycerol, 0.5 mM EDTA and 20 mM 2-mercaptoethanol and removed by centrifugation. The supernatant was centrifuged in 5~20% sucrose gradients containing 1 M NaCl, 0.1 M Tris-HCl (pH 9.5), 0.5 mM EDTA, and 1 mM 2-mercaptoethanol. The active fractions (7.1S) were dialized against 0.01 M Tris-HCl (pH 8.1), 20% glycerol and 2 mM 2-mercaptoethanol.

Determination of DNA Melting Temperatures

Highly polymerized calf thymus DNA (Sigma Type I) at 1 mg/ml in 0.01 M Tris-HCl (pH 7.0), 0.05 M NaCl was sheared in a Virtis "45" homogenizer at a setting of "7" for 20 minutes. Melting profiles were then determined in 0.01 M Tris-HCl (pH 7.0) using 25 $\mu$g/ml of the sheared DNA preparation. The samples were overlaid with heavy mineral oil to prevent evaporation, and the optical density at 260 nm was determined in a Beckman model 25 double-beam spectrophotometer. The reference cuvette always contained the same solution as the sample cuvette minus the DNA. The cuvettes were heated by circulating water through the cuvette holder, and the rate of temperature increase was the same for all experiments (about 0.5°C/min.) The melting temperature was taken to be that point on the melting profile halfway between the plateaus of the least and the greatest optical density.

## Results

Anthracycline Inhibition of $\alpha$ and $\beta$ Polymerases Under $K_m$ Conditions

The incorporation of $^3$H-TTP carried out by the partially purified DNA polymerase $\alpha$ and $\beta$ preparations was determined as described in Materials and Methods under "assays of DNA polymerase activity" except that varied amounts of "activated" DNA were used. More than twelve single determinations were performed for each polymerase preparation. Analysis of the linear LINEWEAVER-BURK plots of the data showed the $K_m$ values for the "activated" DNA for DNA polymerases $\alpha$ and $\beta$ to be 26 and 240 $\mu$g/ml, respectively. The effects of doxorubicin, daunomycin and rubidazone on the DNA polymerase reactions were then determined using these $K_m$ amounts of "activated" DNA.

$\alpha$ Polymerase inhibition: Doxorubicin, daunomycin and rubidazone effectively inhibited $^3$H-TTP incorporation carried out by the $\alpha$ polymerase preparation over a range of concentrations from 0 to 13.6 n moles/ml (Fig. 1, A). Although differences between the effects of individual agents were small, it appeared as though the three agents were effective in the relative order doxorubicin > daunomycin > rubidazone.

$\beta$ Polymerase inhibition: Doxorubicin, daunomycin and rubidazone effectively inhibited $^3$H-TTP incorporation carried out by the $\beta$ polymerase preparation over a range of concentrations from 0 to 128 n moles/ml (Fig. 1, B). The same general order of inhibition (doxorubicin > daunomycin > rubidazone) found for the $\alpha$ enzyme was noted for the $\beta$ enzyme only at the highest level of inhibition. At low concentrations, rubidazone appeared to be more inhibitory than either doxorubicin or daunomycin.

## DNA Melting Temperature Determinations

The effects of doxorubicin, daunomycin and rubidazone on the melting temperature of calf thymus DNA are illustrated in Fig. 2. The melting temperature of the DNA alone was 72.0°C. At a concentration of 5.0 n moles/ml, all three agents significantly increased the melting temperature of the DNA, with increments of 13.6°C (doxorubicin), 12.5°C (daunomycin) and 11.3°C (rubidazone) above the control value.

## Comparative Inhibition of $\alpha$ and $\beta$ Polymerases Using Excess DNA

In order to conduct these experiments, in which inhibition of the DNA polymerase $\alpha$ and $\beta$ preparations could be compared directly, it was necessary to select an amount of DNA substrate which would be at the same concentration in all reaction mixtures while also assuring maximal reaction velocity for both enzymes; 1.6 mg DNA/ml was chosen. This concentration is 6.7 and 66.7 times the $K_m$ amounts of DNA for the $\beta$ and $\alpha$ polymerases respectively.

The inhibitory effects of doxorubicin, daunomycin and rubidazone upon $^3$H-TTP incorporation carried out by both the $\alpha$ and $\beta$ polymerase preparations were compared at drug concentrations from 0 to 640 n moles/ml (Fig. 3). The inhibition observed was directly related to the quantities of drugs, and the effects of all three agents upon the individual enzyme preparations were very similar. However, the $\alpha$ enzyme pre-

Fig. 1. Effects of doxorubicin, daunomycin, and rubidazone on $^3$H-TTP incorporation catalyzed by DNA polymerase $\alpha$(A) or DNA polymerase $\beta$(B).

Assays were performed as described in Materials and Methods except that the $K_m$ amount of "activated" DNA was used for each polymerase preparation (26 and 240 $\mu$g/ml for DNA polymerase $\alpha$ and $\beta$ respectively). The controls (no drugs) for the $\alpha$ and $\beta$ polymerase preparations incorporated approximately 0.04 n mole (30,000 cpm) and 0.08 n mole (60,000 cpm) of $^3$H-TTP respectively.

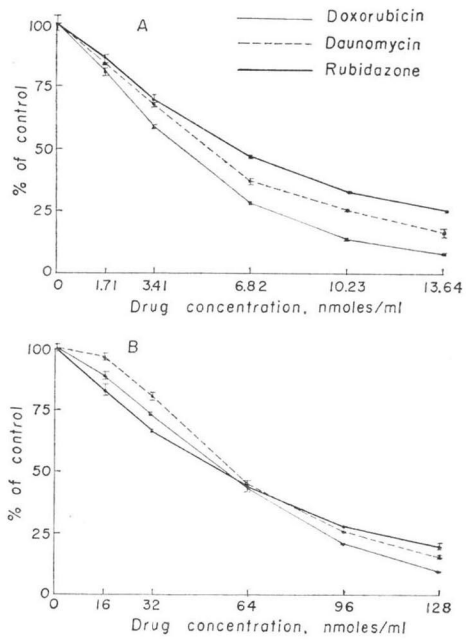
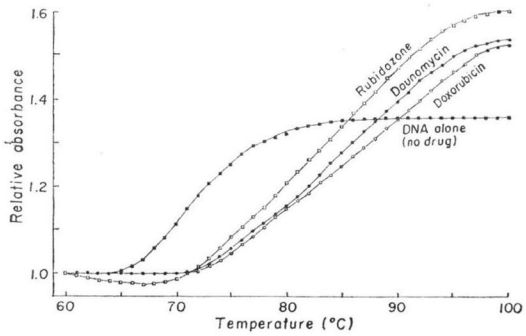



Fig. 2. Influence of doxorubicin, daunomycin, and rubidazone on the melting temperature of DNA.

The amount of DNA used in all experiments was 25 $\mu$g/ml, and the drug concentrations were 5 n moles/ml. Absorbance at 60°C was taken as 1.0 and individual determinations were serially measured at 1.0°C increments and plotted as relative values.



paration was consistently more inhibited at any drug concentration than the $\beta$ polymerase preparation. At a concentration of 640 n moles/ml, the inhibition of the $\alpha$ enzyme was virtually complete for all three drugs.

## Nuclear DNA Polymerase Inhibition

The inhibitory effects of doxorubicin, daunomycin and rubidazone upon $^3$H-TTP incorporation into three types of nuclear preparations were studied, and are illustrated in Fig. 4. Inhibition is dose related, and greatest for the Brij 58, $MgCl_2$-extracted regenerating nuclei, intermediate for the regenerating nuclei, and least for the normal nuclei, with all three agents.

Fig. 3. Effects of doxorubicin, daunomycin and rubidazone on $^3$H-TTP incorporation by the partially purified DNA polymerase $\alpha$(---) and $\beta$(—) preparations.

Assays were as described in Materials and Methods, and the controls (no drugs) for both enzyme preparations incorporated approximately 0.04 n mole of $^3$H-TTP (30,000 cpm).

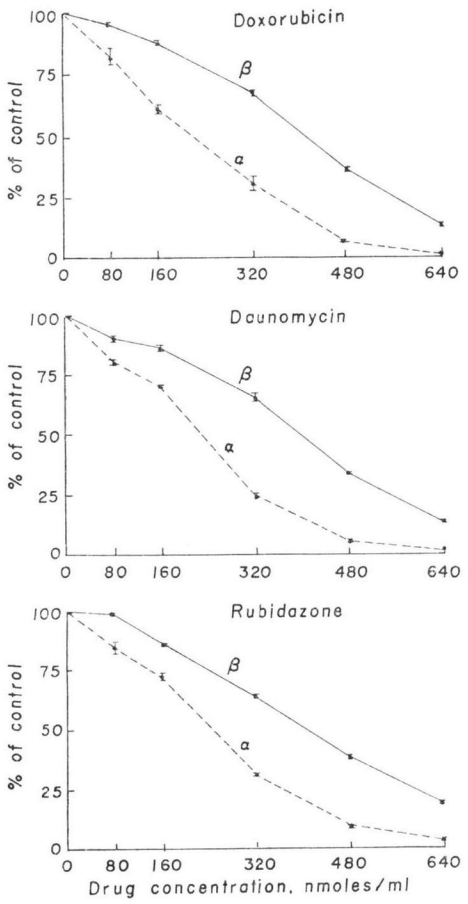
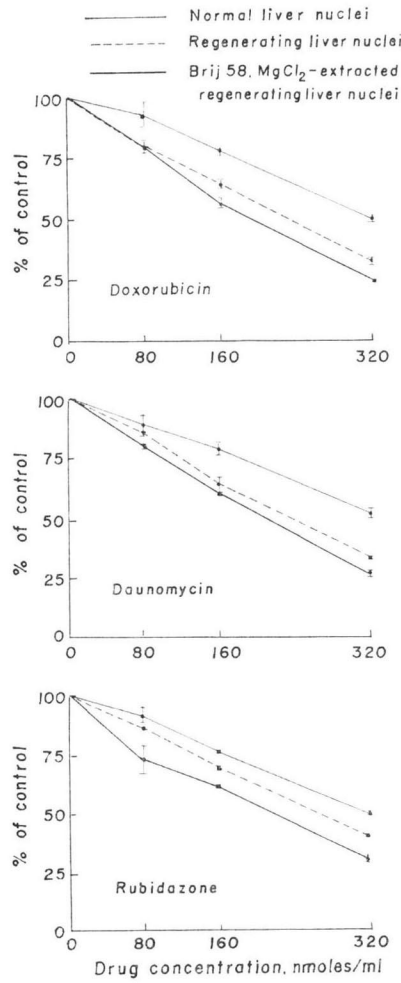



Fig. 4. Inhibitory effects of doxorubicin, daunomycin and rubidazone on $^3$H-TTP incorporation into DNA in the presence of normal, regenerating, and Brij 58, $MgCl_2$-extracted regenerating liver nuclei.

Reaction mixtures were as described in Materials and Methods, and each assay contained $7.5 \times 10^5$ nuclei. The controls (no drugs) incorporated 0.04, 0.15 and 0.10 n mole of $^3$H-TTP for the normal, regenerating and Brij 58, $MgCl_2$-extracted regenerating nuclei, respectively.



## Discussion

The major mechanism of action of the anthracycline anti-tumor antibiotics appears to involve intercalative binding to DNA, with subsequent inhibition of DNA synthesis. Single strand DNA breakage[13~15], blocking of RNA synthesis[24,25] and nucleolar alterations[26] have also been described.

Previously reported studies have demonstrated the ability of doxorubicin and daunomycin to inhibit DNA polymerases from viral[2,17], bacterial[16] and mammalian[2] sources. However, the reported studies have not compared the relative inhibitory effects of the anthracycline antibiotics upon the $\alpha$ polymerase, the presumed replicative enzyme, and the $\beta$ polymerase, the putative repair enzyme, from a single mammalian tissue, as carried out in our experiments.

Studies of the inhibitory effects of doxorubicin, daunomycin and rubidazone upon the partially purified $\alpha$ and $\beta$ polymerases under $K_m$ conditions show a progressive pattern of dose-related inhibition of $^3$H-TTP incorporation in comparison to control values. The inhibitory effect of these three drugs in equimolar concentrations is usually in the order doxorubicin > daunomycin > rubidazone. This order of inhibitory effect is parallel to the diminishing order of melting temperature elevation for the DNA: drug combination, suggesting a relationship between the intercalation of the drugs and the inhibition of the polymerase activities.

It is recognized that the native compounds, doxorubicin, daunomycin and rubidazone undergo chemical alterations *in vivo*, resulting in metabolites of varying activities[27]. It cannot therefore be presumed that the *in vitro* studies described above permit direct extrapolation to the *in vivo* situation or necessarily reflect relative chemotherapeutic activity for these agents *in vivo*.

In order to make it possible to compare directly the effects of the anthracycline antibiotics upon the separate, partially purified $\alpha$ and $\beta$ polymerase preparations, it seemed necessary to define experimental conditions in which the drug: DNA ratio and DNA concentration were the same for both reaction mixtures. It was shown that the $K_m$'s for these two enzymes differ significantly, but direct comparison, under identical conditions, seems essential and also seems most analogous to the *in vivo* situation. A DNA concentration at which optimal activity of both enzymes would be produced was chosen. This DNA concentration represents a slight excess over maximal activity for the $\beta$ enzyme preparation, and a modest excess for the $\alpha$ enzyme preparation. The results clearly show a consistent pattern of preferential $\alpha$ polymerase inhibition under these conditions. For example, for doxorubicin at a concentration of 320 n moles/ml, the $\beta$ enzyme activity is only $32.0 \pm 1.0\%$ inhibited, while the $\alpha$ activity is $69.0 \pm 2.7\%$ inhibited. This preferential inhibition is all the more striking when it is recognized that the substrate DNA concentration used in these experiments was 61 times the $K_m$ amount for the $\alpha$ polymerase but only 6.7 times the $K_m$ amount for the $\beta$ polymerase. Under these circumstances it would have been predicted that, enzyme substrate requirements being similar, the $\beta$ enzyme would be more inhibited than the $\alpha$ enzyme at any given drug concentration. The contrary finding of $\alpha > \beta$ inhibition therefore is reinforced, and suggests that the polymerizing activity carried out by the $\alpha$ polymerase is much more readily inhibited than the $\beta$ polymerase activity at equal drug concentrations.

The experiments carried out with the isolated liver nuclei were intended to make it possible to compare directly the inhibitory effects of doxorubicin, daunomycin and rubidazone upon nuclei which contained predominantly $\beta$ activity (normal nuclei) or both $\alpha$ and $\beta$ activity, with $\alpha$ actively predominating (regenerating nuclei), or mainly $\alpha$ activity (Brij 58, $MgCl_2$-extracted regenerating nuclei). As would have been predicted from the experiments with partially purified enzyme preparations, the agents doxorubicin, daunomycin and rubidazone consistently inhibited the different types of nuclei in the order: Brij 58, $MgCl_2$- extracted ($\alpha$) > regenerating ($\alpha$ and $\beta$) > normal ($\beta$). These data suggest that the preferential inhibition of DNA polymerase $\alpha$ which was found in both partially purified enzyme preparations and intact nuclei, may also obtain in whole cells.

Recent reclassification of the chemotherapeutic agents on a functional basis, measuring cytotoxicity based on tumor cell and normal colony-forming assays, has resulted in the division of agents into three groups: (1) Phase Specific, (2) Cycle Non-Specific, (3) Cycle Specific[28]. The Cycle Specific group has been defined as a group of agents which, like the Cycle Non-Specific group, kill a fixed frac-

tion of all cells present, with each dose increment, regardless of whether the cells are involved in the mitotic cycle or not. However, the Cycle Specific group differs in that this indiscriminate cell kill shows some preference for tumor cells, whereas the cytotoxic effects of the Cycle Non-Specific agents do not show this relative selectivity[29]. Moreover, it has been shown that this cell kill preference for actively mitotic tumor cells in comparison to normal cells can be obliterated if the relatively quiescent normal cell population is stimulated into mitotic activity[30] demonstrating that the selectivity involved is dependent upon the level of mitotic activity rather than specificity of the drug for a particular normal or tumor cell type.

When cells enter the replicative cycle, their content of $\alpha$ polymerase rises substantially, while their content of $\beta$ polymerase remains unchanged[20,21]. Under these circumstances, cells involved in the mitotic cycle would be more susceptible to an agent which preferentially inhibits the $\alpha$ polymerase. We therefore suggest that a preferential $\alpha$ polymerase inhibition may underlie, in part or in whole, the cell Cycle Specific cytotoxic effects of the agents doxorubicin, daunomycin and rubidazone, and may explain their reclassification into this subcategory.

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