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EFFECT OF SANGIVAMYCIN AND XYLOSYLADENINE ON THE SYNTHESIS AND METHYLATION OF POLYSOMAL RIBONUCLEIC ACID IN EHRLICH ASCITES CELLS *IN VITRO*

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Abstract—The pyrrolopyrimidine, sangivamycin, and the adenosine analog, xylosyladenine, were examined for their effects on the synthesis and methylation of polysomal RNA in Ehrlich ascites tumor cells *in vitro*. The synthesis of non-polyriboadenylic acid (non-poly (A) −) and poly(A)-containing RNA was inhibited 50 per cent at concentrations of 7×10^{-6} M and 3×10^{-6} M xylosyladenine, respectively, when adenosine deaminase was inhibited with 2′-deoxycoformycin. Sangivamycin inhibited the synthesis of non-poly(A)- and poly(A)RNA by 50 per cent at concentrations of 5×10^{-5} M and 2×10^{-5} M respectively. Electrophoretic separation of non-poly(A)RNA into rRNA and tRNA indicated that the inhibitory effects of both drugs were more pronounced on 28S than on 18S rRNA, and that xylosyladenine but not sangivamycin inhibited the synthesis of tRNA. Assessment of the effects of both analogs on the methylation of polysomal RNA revealed that xylosyladenine inhibited the methylation of non-poly(A)- and poly(A)RNA, while sangivamycin only weakly affected the latter species of RNA. Base methylation of the affected species of RNA was inhibited slightly more than 2′-*O*-methylation by both drugs. These results indicate that sangivamycin is a more selective inhibitor of polysomal RNA in comparison to xylosyladenine under conditions where adenosine demainase is not a limiting factor.

Several studies have indicated that the therapeutic activity of adenosine analog antitumor agents such as XA† can be enhanced by prior inhibition of adenosine deaminase with dCF, a tight-binding inhibitor [1–3]. Utilization of this approach in cancer chemotherapy has been reviewed recently [4]. An alternative pharmacological approach can be achieved by the utilization of drugs that are inherently resistant to inactivation by adenosine deaminase as exemplified by the pyrrolopyrimidine, sangivamycin [5]. The latter drug possesses significant antitumor activity *in vivo* against L1210 and colon 37 tumors (J. Plowman, personal communication) and against Sarcoma 180 cells in tissue culture [6], and it is one of the few adenosine analogs to reach clinical trials [7].

Both XA and sangivamycin are effective inhibitors of nuclear RNA synthesis in L1210 cells *in vitro* [8, 9]. The effects of XA on transcription however, are by no means specific since this drug is an equally potent inhibitor of nuclear RNA methylation [10]. In the latter instance, it is metabolized to an analog of *S*-adenosyl-L-methionine [11] and serves as an effective inhibitor of *S*-adenosyl-L-methionine synthesis [12]. In contrast, sangivamycin has little or no effect on the methylation of nuclear RNA in L1210 cells [9]. Methylation reactions are crucial for the maturation of nuclear rRNA, mRNA and tRNA to their cytoplasmic counterparts [13–15]. Drugs producing *less* inhibition of methyltransferase reactions might be expected to serve as more specific chemotherapeutic agents since other metabolic processes such as adrenergic receptor transmission [16] and cellular differentiation and gene function [17] are dependent upon methyltransferase reactions.

In the present report, we wish to present data regarding the specificity of action of the adenosine analogs, XA and sangivamycin, on cytoplasmic RNA from polysomes of Ehrlich ascites tumor cells. Polysomal RNA was fractionated by affinity chromatography into non-poly(A)RNA (rRNA and tRNA) and poly(A)RNA (mRNA), and the effects of the two analogs on the synthesis and methylation of these species of RNA were determined.

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† Abbreviations: XA, xylosyladenine [9-(β-D-xylofuranosyl)adenine]; non-poly(A)RNA, non-polyriboadenylic acid-containing RNA; poly(A)RNA, polyriboadenylic acid-containing RNA; dCF, 2′-deoxycoformycin [(R)-3-(2′-deoxy-β-D-erythro-pentofuranosyl)-3,6,7,8-tetrahydroimidazo [5, 4-d] [1,3]diazepin-8-ol]; sangivamycin, 4-amino-7-(β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine-5-carboxamide; IC_{50}, median inhibitory concentration; and SDS, sodium dodecylsulfate.

MATERIALS AND METHODS

Materials. Sangivamycin and XA were obtained from the Natural Products Branch and Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD. [U-^{14}C]Uridine (522 mCi/mmole) and [5-methyl-^{3}H]-L-methionine (80 Ci/mmole) were purchased from the New England Nuclear Corp., Boston, MA. Poly(U)Sepharose was obtained from Pharmacia Fine Chemicals, Piscataway, NJ.

Animals. Ehrlich ascites tumor cells were maintained in NIH Swiss mice at an i.p. inoculum of 10^5 cells/0.1 ml of Hanks' balanced salt solution. Cells were harvested 1 week after inoculation and washed once with RPMI 1630.

Incubations. Ehrlich cells were incubated at 37° in a shaking water bath at 100 rpm and consisted of: 250 ml of RPMI 1630 medium containing 10% heat-inactivated newborn calf serum, either dCF and XA dissolved in water or sangivamycin dissolved in dimethylformamide (final concentration 0.1%, v/v), 5×10^8 cells, and either 25 μCi [^{14}C]uridine (522 mCi/mmole) or 500 μCi [^{3}H-methyl]methionine (80 Ci/mmole) and 25 μCi [^{14}C]uridine. In all experiments utilizing XA, cells were preincubated for 15 min with 1×10^{-6} M dCF before addition of XA. Control incubations contained either an equivalent volume of water or dimethylformamide. Subsequently, cells were incubated with either XA or sangivamycin for 30 min followed by labeling with radioactive precursors for 1 hr. dCF had no effect on either the synthesis or methylation of polysomal RNA, and the results obtained were identical to either untreated cells [8, 12] or cells incubated with 0.1% dimethylformamide [9].

RNA extractions. After incubation, cells were centrifuged at 400 *g* for 20 min and washed once with incubation medium. Polyribosomes were prepared as described previously [18]. Total polysomal RNA was extracted by continuous vortexing with 1 vol. of 0.5% SDS: 0.1 M Tris–HCl (pH 9.0), 0.5 vol. of phenol mixture (phenol–*m*-cresol–H_2O, 7:2:2, by vol., containing 0.1% 8-hydroxyquinoline), followed by 0.5 vol. of chloroform. The emulsion was clarified by centrifugation at 12,000 *g* for 10 min and the upper phase removed and precipitated with 3 vol. of 95% ethanol at −20° for 3 hr. Poly(A)RNA was isolated from total polysomal RNA by poly(U)Sepharose chromatography as described previously [18].

Electrophoresis. Poly(A)RNA was electrophoresed in 2% agarose gels containing: 15 mM iodoacetate, 6 M urea and 0.4 M Tris: 0.02 M Na acetate: 0.033 M acetic acid: 0.01 M EDTA (pH 7.4) as described by Locker [19]. Gels were sliced into 2-mm sections, dissolved in 70% perchloric acid, and mixed with 10 ml of Aquasol (New England Nuclear). Radioactivity was determined in a Searle Mark III liquid scintillation spectrometer.

DEAE Sephadex chromatography. Alkaline hydrolysis of non-poly(A)- and poly(A)RNA and ion-exchange chromatography on DEAE Sephadex were carried out as described previously [10]. In this procedure, alkaline hydrolysates of RNA are separated mainly into mononucleotide and dinucleotide fractions having a net charge of −2 and −3 respectively.

RESULTS

Concentration-dependent effects. Initial experiments were carried out to determine the concentration dependence of the inhibition of methylation and synthesis of non-poly(A)- and poly(A)RNA isolated from polyribosomes of Ehrlich ascites cells (Figs. 1 and 2). Under conditions where adenosine deaminase was completely inhibited by preincubation of cells for 15 min with 1×10^{-6} M dCF, XA inhibited the synthesis and methylation of non-poly(A)- and poly(A)RNA to approximately the same degree (Fig. 1). dCF alone did not affect either the synthesis or methylation of polysomal RNA as observed previously for nuclear RNA and *S*-adenosyl-L-methionine synthesis [8, 12, 20]. Sangivamycin, although producing a pronounced inhibition of polysomal RNA synthesis, was a weaker inhibitor than XA (Fig. 2). The IC_{50} for non-poly(A)- and poly(A)RNA was 7-fold greater than obtained with XA; in contrast to XA, however, sangivamycin did not affect the methylation of non-poly(A)RNA and was a 20-fold weaker inhibitor of poly(A)RNA methylation. The synthesis of poly(A)RNA was about twice as sensitive as non-poly(A)RNA to inhibition by both drugs.

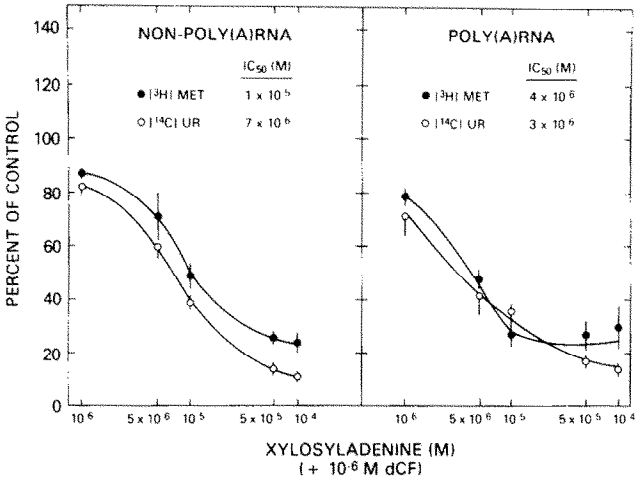

Fig. 1. Effect of XA concentration on the synthesis and methylation of total RNA from polysomes of Ehrlich ascites cells. Cells (5×10^8 cells/100 ml) were preincubated for 15 min with 1×10^{-6} M dCF and then incubated with XA for 30 min. RNA was subsequently labeled for 1 hr after the addition of 500 μCi [^{3}H-methyl]methionine and 25 μCi [^{14}C]uridine and extracted from isolated polysomes as described in Materials and Methods. Each value is the mean ± S.E. of five to ten determinations.

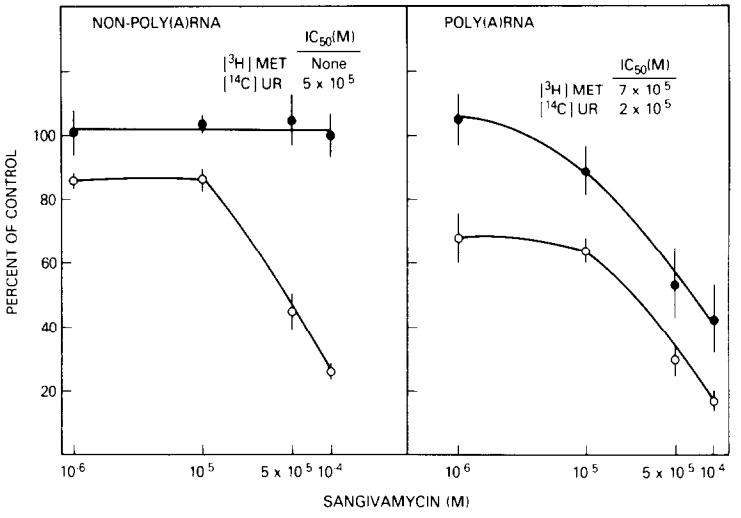

Fig. 2. Effect of sangivamycin concentration on the synthesis and methylation of total RNA from polysomes of Ehrlich ascites cells. Cells were preincubated for 15 min with 100 μl dimethylformamide (equivalent to the volume of dCF used in Fig. 1) and then incubated with sangivamycin for 30 min. RNA was subsequently labeled as described in Fig. 1. Each value is the mean ± S.E. of five to twelve determinations. ●, [^{3}H]Met; ○, [^{14}C]UR.

Electrophoresis. Electrophoretic separation of non-poly(A)RNA indicated that it consisted mainly of 28S and 18S rRNA and tRNA (4S RNA) (Fig. 3A). Concentrations of XA and sangivamycin approximating their IC_{50} values were used throughout these experiments. XA in the presence of dCF inhibited 28S, 18S and 4S RNA by 63, 42 and 47 per cent respectively (Fig. 3B). In contrast, sangivamycin inhibited 28S, 18S and 4S RNA by 52, 39 and 0 per cent respectively. dCF treatment alone had no effect on the electrophoretic profiles of either non-poly(A) or poly(A)RNA.

Analysis of poly(A)RNA from control cells indicated that it was heterodisperse with a median size of about 13S (Fig. 4A). XA and sangivamycin inhibited incorporation of [^{14}C]uridine into this RNA species by 60 and 70 per cent respectively (Fig. 4, B and C).

DEAE Sephadex-urea chromatography. Alkaline hydrolysates of non-poly(A)- and poly(A)RNA double-labeled with [^{3}H-methyl]methionine and [^{14}C]uridine were separated into mononucleotide (−2 charge), dinucleotide (−3 charge) and oligonucleotide (−5 to −6 charge) fractions by ion-exchange chromatography (Figs. 5 and 6). The methylated mononucleotide fraction represents base methylation while the methylated dinucleotide fraction is indicative of 2′-*O*-methylation which confers resistance to endonucleases hydrolyzing the 3′,5′-phosphodiester bond. Methylation in oligonucleotides in poly(A)RNA presumably represents base and 2′-*O*-methylated sequences in the 5′-terminus or "cap". XA inhibited the methylation of the mono- and dinucleotide fractions arising from non-poly(A)RNA by 48 and 41 per cent respectively (Fig. 5B); in contrast, sangivamycin did not inhibit the

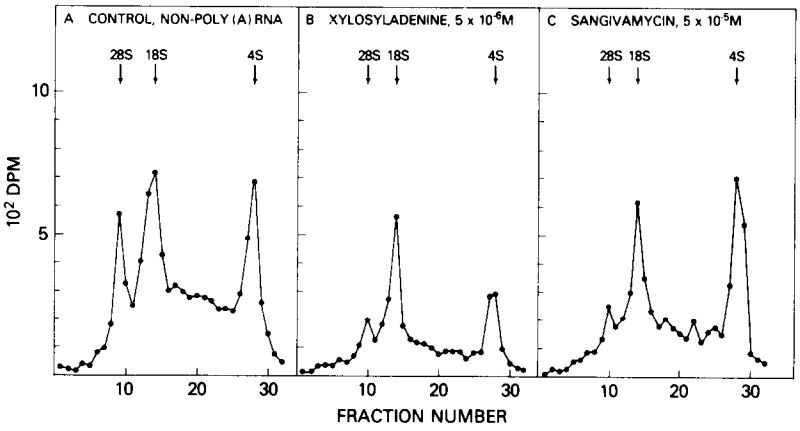

Fig. 3. Agarose-urea electrophoresis of non-poly(A)RNA from polysomes of Ehrlich ascites cells. Cells were treated with either dCF + XA or sangivamycin as described in Figs. 1 and 2. RNA was labeled for 1 hr with 25 μCi [^{14}C]uridine, and extracted and electrophoresed as described in Materials and Methods. Control RNA was obtained from cells treated with 0.1% dimethylformamide, but it was identical to that obtained from cells treated with 1×10^{-6} M dCF alone or an equivalent volume of water.

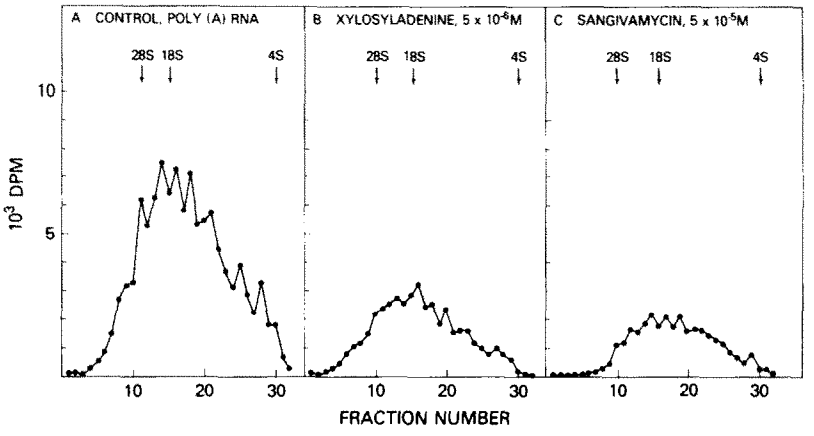

Fig. 4. Agarose-urea electrophoresis of poly(A)RNA from polysomes of Ehrlich ascites cells. Cells were treated as described in the legend of Fig. 3.

methylation of this species of RNA (Fig. 5C). The radioactivity recovered in the mononucleotide fraction labeled with [^{14}C]uridine was reduced 53 per cent by XA and 59 per cent by sangivamycin. DEAE Sephadex chromatography of alkaline hydrolysates of poly(A)RNA indicated that inhibition of methylation was predominantly in the mononucleotide fraction (Fig. 6). Methylations of the mono-, di- and oligonucleotide fractions were each inhibited by 65, 51 and 51 per cent, by XA, and by 35, 22 and 9 per cent, respectively, by sangivamycin. Labeling of the mononucleotide fraction with [^{14}C]uridine was reduced 58 and 61 per cent by XA and sangivamycin respectively. Thus, XA in the presence of dCF produced slightly more inhibition of base methylation than of 2′-*O*-methylation in non-poly(A)- and poly(A)RNA, whereas sangivamycin showed the same selectivity for poly(A)RNA. In both instances, inhibition of RNA synthesis, as assessed by the incorporation of [^{14}C]uridine into the mononucleotide fraction, was greater than the reduction in methylation of this fraction.

DISCUSSION

The mechanism of action of XA is believed to arise, in part, from its ability to nonselectively inhibit transcription of all species of nuclear RNA [9] via its conversion to the active metabolite, XA-5′-triphosphate [21]. Recently, XA was also found to produce equally profound effects upon the methylation of nuclear RNA in L1210 cells [10]. The latter action results from its ability to impair the synthesis of *S*-adenosyl-L-methionine [12] by serving as an alternative substrate for ATP for *S*-adenosyl-L-methionine synthetase [11]. In the present study, the selectivity of XA on the methylation and synthesis of cytoplasmic RNA was determined. The IC_{50} for inhibition of polysomal RNA synthesis was of the same order of magnitude as observed previously for total nuclear RNA in L1210 cells [8]. Inhibition of methylation of non-poly(A)- and poly(A)RNA by XA in the presence of dCF was of the same degree as the inhibition of *S*-adenosyl-L-methionine synthesis [12]. Therefore, it appears that the concerted

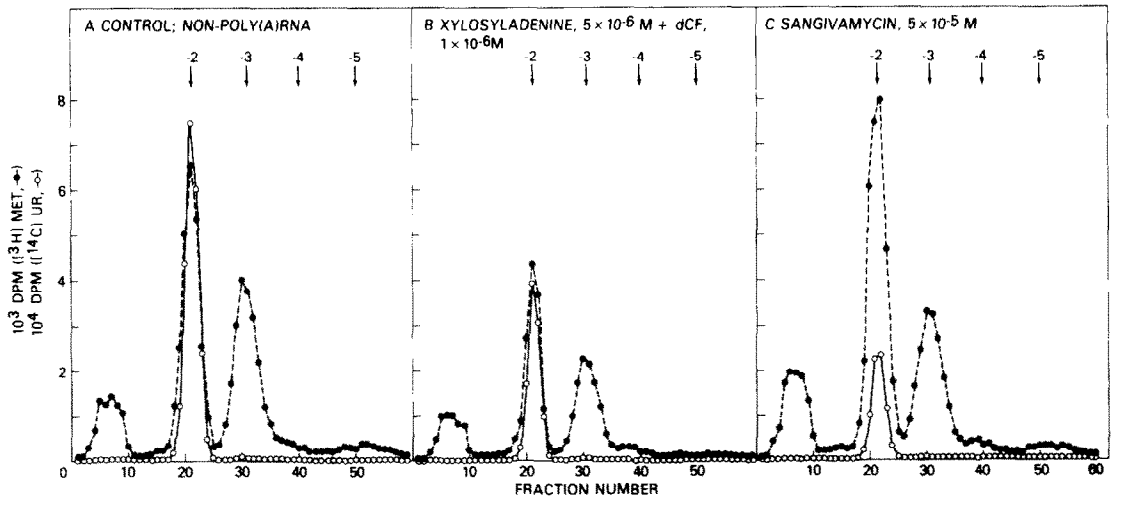

Fig. 5. DEAE Sephadex-urea chromatography of alkaline hydrolysates of non-poly(A)RNA from polysomes of Ehrlich ascites cells. Cells were treated as described in the legend of Fig. 3. Alkaline hydrolysates of non-poly(A)RNA were prepared as described previously [10] and adsorbed to DEAE Sephadex containing 20 mM Tris–HCl (pH 7.6):7 M urea. Mononucleotide (−2 charge) and dinucleotide (−3 charge) fractions were eluted with a linear gradient of 0.1 to 0.7 M NaCl in 20 mM Tris–HCl (pH 7.6):7 M urea.

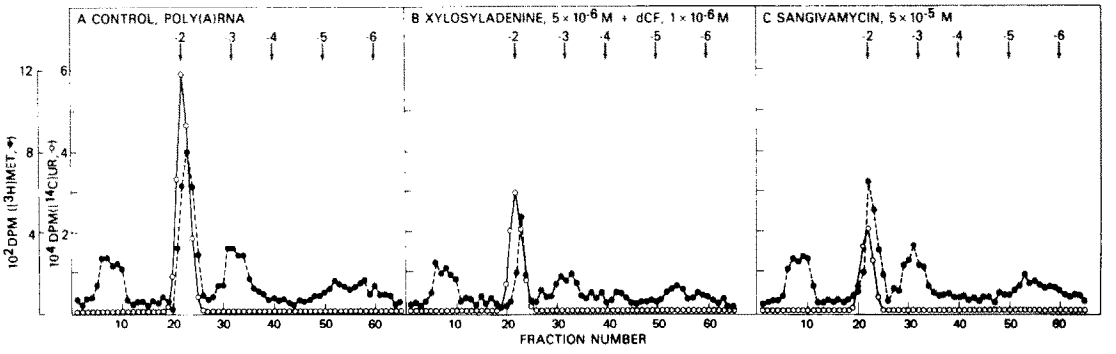

Fig. 6. DEAE Sephadex-urea chromatography of alkaline hydrolysates of poly(A)RNA from polysomes of Ehrlich ascites cells. Cells were treated as described in the legend of Fig. 3. Alkaline hydrolysates were prepared as described previously [10] and chromatographed as stated in the legend of Fig. 5.

action of this drug is upon both the synthesis and methylation of polysomal and nuclear RNA.

In contrast, sangivamycin is a weak inhibitor of methylation of nuclear RNA [9] and polysomal RNA, but it does possess significant inhibitory effects on the transcription of nuclear and cellular RNA [6, 9]. The IC_{50} of sangivamycin for nuclear RNA synthesis in L1210 cells [9] was equivalent to its inhibitory effect on polysomal RNA. However, the transcription of rRNA, particularly 28S rRNA, and poly(A)RNA was selectively inhibited while the synthesis of tRNA was unaffected. This effect is undoubtedly mediated via sangivamycin 5'-triphosphate since this metabolite inhibits *Escherichia coli* RNA polymerase activity *in vitro* [22], and the parent drug is incorporated into RNA of normal tissues of mice [23] and S-180 cells in tissue culture (P. S. Ritch and R. I. Glazer, unpublished results). Sangivamycin did not affect the methylation of non-poly(A)RNA but proved to be a weak inhibitor of poly(A)RNA base methylation. Therefore, it appears that this antibiotic is a more specific inhibitor of transcription and that the resultant reduction in methylation of poly(A)RNA is a consequence of impaired transcription rather than the primary effect of this drug.

The greater sensitivity of 28S rRNA, compared to 18S rRNA, to the inhibitory effects of XA and sangivamycin suggests that these drugs may produce premature termination of transcription of ribosomal precursor RNA. That hypomethylation is probably not involved with this effect is suggested by the fact that sangivamycin does not impair the methylation of rRNA in contrast to XA, but it does produce the same differential effect on 28S rRNA as XA. Since the latter species of rRNA is transcribed toward the 3'-terminus of ribosomal precursor RNA [24], it would be expected that premature termination of transcription would preferentially reduce the synthesis of 28S rRNA.

In summary, it appears that there are distinguishing qualitative differences between the pyrrolopyrimidine, sangivamycin, and the adenosine analog, XA, in their effects on the synthesis and methylation of cytoplasmic RNA. These differences in mode of action may serve as a valuable framework for designing other nucleoside analogs that are more specific in their mode of action and are not compromised in their therapeutic activity by adenosine deaminase.

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