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ARABINOSYL NUCLEOSIDES—XXXIII. METABOLISM OF 9-β-D-ARABINOFURANOSYLADENINE IN YEAST CELLS

DAN-KARL SCHUSTER,* RUDOLF K. ZAHN,* HANS STERNBACH,† MIROSLAV J. GAŠIĆ,‡ RUDOLF BEYER* and WERNER E. G. MÜLLER*§

*Institut für Physiologische Chemie, Universität, Duesbergweg, 6500 Mainz, Federal Republic of Germany, †Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, Hermann-Rein-Str. 3, 8400 Göttingen, Federal Republic of Germany and ‡Faculty of Sciences, Department of Chemistry, University, Beograd, Yugoslavia

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Abstract—Growth of *Saccharomyces cerevisiae* is not affected by 9-β-D-arabinofuranosyladenine (β-ara-A) up to concentrations of 100 μM. Analytical studies revealed that β-ara-A is taken up by the yeast but is not intracellularly phosphorylated to the corresponding nucleotides; 27 per cent of the intracellular β-ara-A is deaminated to 9-β-D-arabinofuranosylhypoxanthine. However, incubation of the cells with ara-AMP results in a strong inhibition of cell proliferation with an ED_{50} concentration of 8.3 μM. The ara-AMP-caused inhibition of cell proliferation can be abolished by coincubation with deoxyadenosine. The latter observation together with results from incorporation studies indicates that ara-AMP affects cell growth via inhibition of DNA synthesis. *In vitro* experiments with partially purified adenosine kinase from yeast revealed that β-ara-A and eight further aranucleosides as well as three deaminase inhibitors (diazepin derivatives) are either not substrates or poor ones for this enzyme.

Only a very limited number of base and nucleoside analogues of nucleic acid metabolism have been found to inhibit DNA or RNA synthesis in yeast [1]. This fact is primarily due to the impermeability of the cell walls of yeasts to some of the inhibitors found to be active in other eukaryotic cell systems [2]. To overcome the permeability barrier existing for some of the bases or nucleosides (e.g. Thy and dThd), the 5′-monophosphate derivatives, especially those of deoxyribonucleosides, have been successfully applied, and they are taken up by this organism [3–5]. To our knowledge, up to now no nucleotide analogue has been described which was found to inhibit either DNA or RNA synthesis in yeast.

In the present study the metabolism of 9-β-D-arabinofuranosyladenine (β-ara-A)‖, a potent antitumor and antiviral antimetabolite [6, 7] in yeast cells is described. Besides β-ara-A a series of other aranucleosides and three adenosine deaminase inhibitors, coformycin [(R)-3-(D-erythro-pentofuranosyl)-3,6,7,8-tetrahydroimidazo (4,5-*d*) (1,3) diazepin-8-(R)-ol; Ref. 8], isocoformycin [3-β-D-ribofuranosyl-3,6,7,8-tetrahydroimidazo (4,5-*d*) (1,3) diazepin-7-of; Ref. 9] and 2′-deoxycoformycin [(R)-3-(2-deoxy-β-D-erythropentofuranosyl) - 3,6,7,8-tetrahydroimidazo (4,5-*d*) (1,3) diazepin-8-ol; Ref. 10] were studied with regard to their substrate specificity for adenosine kinase.

MATERIALS AND METHODS

Organism. Saccharomyces cerevisiae (strain 43) was kindly supplied by Prof. F. Radler (Institut für Mikrobiologie und Weinforschung, University, Mainz).

Culture conditions. In all experiments the yeast was grown in YEP-Medium (1% yeast extract, 2% peptone and 2% glucose) at 30° with forced aeration. The cell concentration was determined either turbidimetrically (at 578 nm) or optically (in a haemocytometer). The number of doublings was determined as described previously [11].

Incorporation studies. To measure RNA synthesis, [^{3}H]adenine was used as tracer. This base is first incorporated into the purine nucleotides and then into nucleic acids [12]. More than 95 per cent of the acid-insoluble radioactivity was found to be alkali soluble, which means that this incorporation method measures primarily RNA synthesis. The measurement of DNA synthesis was based on a modification of the method of Roodyn *et al.* [13]. Cells were labelled with [^{3}H]uracil and extracted in 1 N KOH for 18 hr at 37°. The residue was collected on 50 mμ HA-millipore filters. More than 90 per cent of this residue is in DNA [14]. The usual thymidine precursor for determination of DNA synthesis cannot be used with yeast because it is not taken up by

§ To whom correspondence should be addressed.

‖ Abbreviations used: β-ara-A, 9-β-D-arabinofuranosyladenine; ara-AMP, ara-ADP and ara-ATP, 9-β-D-arabinofuranosyladenosine-5′-mono-, di- and triphosphate; α-ara-A, 9-α-D-arabinofuranosyladenine; ara-6MP, 9-β-D-arabinofuranosyl-6-mercaptopurine; β-ara-Hx, 9-β-D-arabinofuranosylhypoxanthine NMP, nucleoside 5′-monophosphate.

The purines, the nucleosides and the nucleotides were abbreviated according to the recommendations of the Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **15**, 203 (1970)).

the cells. For the incorporation studies (assay volume: 5 ml) exponentially growing cells at 1×10^7 cells/ml were supplemented with 0, 10 or 100 μM ara-AMP 30 min prior to the addition of the labelled precursors, 25 μCi[^{3}H]adenine or 25 μCi [^{3}H]uracil. The incorporation with the precursors was continued for 60 min. Samples of 5 ml were analysed for acid-insoluble radioactivity.

Intracellular fate of β-ara-A. Cultures (20-ml) containing 1×10^7 logarithmically growing cells/ml were incubated (60 min; 30°) with 100 μCi (= 0.23 μM) of [^{3}H] β-ara-A. After incubation, the cells were harvested and subsequently washed three times with 0.9% NaCl solution by centrifugation (6000 *g*; 3 min; 0°). The cellular pellet was extracted with 60% methanol. The methanol-soluble fraction was analysed first by ion-exchange column chromatography on QAE-25 Sephadex [15]. Over 95 per cent of the radioactivity applied to the column was recovered by elution with 0.1 M NH_4HCO_3. This fraction was subsequently evaporated, dissolved in distilled water and chromatographed in an ascending system on cellulose F plates (Merck) with the following system [16]: ethanol: ammonia (25%): chloroform (50:5:50); the different R_f values are: β-ara-A, 0.51; β-ara-Hx, 0.23; ara-AMP, 0. The radioactivity was spotted as described [17].

Enzymes. The adenosine kinase (EC 2.7.1.20) was isolated and purified from dried brewer's yeast [18]. The specific activity was determined to be 0.36 U/mg protein (16 mg/ml); one unit of enzyme activity catalyses the phosphorylation of one μmole Ado/min [18].

Assay of adenosine kinase. This method was based on the decrease in absorbance at 340 mμ in a coupled multienzyme system [19]. The assay (volume, 2 ml) contained 200 mM Tris–HCl (pH 7.65), 50 mM KCl, 0.5 mM $MgCl_2$, 0.25 mM phosphoenolpyruvate, 0.2 mM NADH, 0.7 mM ATP, 50 μg lactate dehydrogenase, 100 μg pyruvate kinase, 50 μg myokinase and 10 μl of adenosine kinase. After a preincubation period (at 25°) for 5 min, 1–50 μM adenosine (or a nucleoside analogue which was added in concentrations up to 1 mM) was added and the reaction (at 25°) was followed by the change in absorption at 340 nm. The Michaelis constant (K_m), the maximum reaction velocity (V_{max}), the inhibitor constant (K_i), the apparent value of K_m (K_p) and the apparent value of V_{max} (V_p) in the presence of an inhibitor were determined according to Lineweaver *et al.* [20].

Miscellaneous. Protein was determined according to the method of Lowry *et al.* [21].

Chemicals. The following materials were obtained: ATP, Ado, phosphoenolpyruvate, NADH, lactate dehydrogenase (from rabbit muscle, sp. act. 1.14 U/mg), myokinase (from rabbit muscle) and pyruvate kinase (from rabbit muscle) from Boehringer, Mannheim (F.R.G.); [8-^{3}H]adenine (sp. act., 7.3 Ci/mmole), [2-^{3}H]uracil (sp. act., 4.9 Ci/mmole) and [2,8-^{3}H] β-ara-A (sp. act., 22.0 Ci/mmole) from The Radiochemical Centre, Amersham (U.K.).

We are greatly indebted to Fa. H. Mack, Illertissen (F.R.G.) for the generous gifts of ara-U, ara-C, ara-T, ara-Hx, β-ara-A, α-ara-A, ara-6MP and ara-AMP. Ara-G was a gift of the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A. We thank Dr. A. J. Glazko (Parke, Davis & Co., Detroit, MI, U.S.A.) for the sample of deoxycoformycin. Coformycin and iso-coformycin were kindly supplied by Prof. H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan).

RESULTS

Inhibition of yeast cell growth

Logarithmically growing yeast cells were incubated in the presence of β-ara-A and α-ara-A up to concentrations of 100 μM. Cell proliferation was followed during an incubation period of 20 hr. The results revealed (Fig. 1) that these nucleosides had no effect on the proliferation rate.

In a separate series of experiments the influence of ara-AMP on yeast cell growth was studied. It was found that this nucleotide reduces cell proliferation considerably (Fig. 1). At a concentration of 10 μM ara-AMP the growth was inhibited by 53 per cent and at 100 μM by 77 per cent. In dose–response experiments under identical conditions described in the legend to Fig. 1, an ED_{50} concentration (the concentration which induces a 50 per cent inhibition of cell growth) of 8.3 ± 0.7 μM was determined.

In incorporation studies with the precursors [^{3}H]uracil and [^{3}H]adenine, the influence of ara-AMP on macromolecular synthesis was analysed. As outlined in Materials and Methods the uracil label

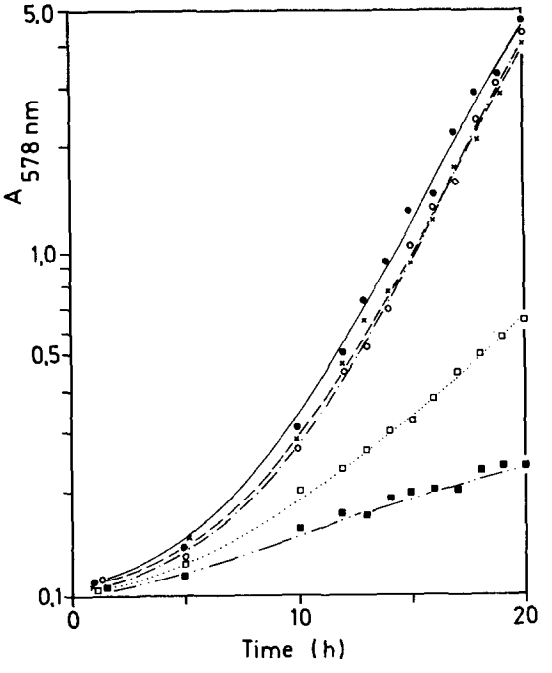

Fig. 1. Dependence of the growth of *S. cerevisiae* cells on addition of ara-A and ara-AMP. The logarithmically growing cultures were supplemented with either 100 μM α-ara-A (×—·—×), 100 μM β-ara-A (○ - - - ○), 10 μM ara-AMP (□·····□) or 100 μM ara-AMP (■—·—■); controls (without ara-A) (●——●). The aranucleosides were added at time 0. Cell growth is measured at the absorbance of the culture at 578 nm (an $A_{578\,nm}$ value of 4.9 corresponds to 1×10^8 cells per ml). Each value comes from 10 parallel assays, and the S. D. is less than 10 per cent.

Table 1. Abolition of the ara-AMP-caused inhibition of cell growth by coincubation with deoxyadenosine*

Ara-AMP (μM)	Deoxyadenosine (μM)	Cell concentration after incubation (cells $\times 10^7$/ml)	Cell doublings	Increase of the number of doubling steps
0	0	5.36	6.7	
10	0	1.91	5.3	
100	0	0.12	1.3	
0	100	5.31	6.7	
10	100	4.62	6.5	1.2
100	100	2.35	5.6	4.3

* The experiments were performed for 12 hr with logarithmically growing cultures at a cell concentration of 5×10^7 cells/ml. The degree of cell proliferation is expressed in doubling steps.

can be used for determination of DNA synthesis after a pretreatment of the acid-insoluble material with alkali and adenine as tracer to measure RNA synthesis. In the absence of ara-AMP the incorporation rate of [^{3}H]uracil into DNA was found to be 7500 ± 620 dpm per 10^7 cells and the one of [^{3}H]adenine into RNA $64{,}200 \pm 5100$ dpm per 10^7 cells. Under these cytostatic concentrations of 10 and 100 μM of ara-AMP this incorporation rate of uracil into DNA is reduced by 35 or 58 per cent respectively, while the adenine incorporation into RNA is not influenced at all.

Changes of incorporation of precursors into nucleic acids do not always reflect the absolute values for the extent of DNA or RNA synthesis (because possible alterations of the intracellular precursor pools in response to drug treatment could occur); therefore a second approach was used to determine the influence of ara-AMP on DNA synthesis. The yeast cells were incubated in the presence of cytostatic concentrations of ara-AMP together with deoxyadenosine, the physiological DNA precursor, which is taken up by the yeast cells [5]. As summarized in Table 1, the ara-AMP-induced inhibition of cell proliferation could be abolished by coincubation with deoxyadenosine; the cell doublings increase from 5.3 (1.3) in the absence of deoxyadenosine to 6.5 (5.6) in the absence of the natural nucleoside. Experiments coincubating adenosine with ara-AMP revealed no measureable reduction in inhibitory potency of the ara-AMP (data not shown).

Biochemical mode of action

Intracellular phosphorylation of ara-A. From experiments with prokaryotic and eukaryotic biological models [6, 7], it is known that ara-A inhibits nucleic acid-synthesizing enzyme systems only after it has been phosphorylated to ara-AMP, ara-ADP and ara-ATP. To clarify whether the resistance of yeasts to ara-A is due to an inability of their enzyme system to phosphorylate this nucleoside analogue, the metabolic fate of β-ara-A was studied. Yeast cells were incubated for 1 hr in the presence of [^{3}H]-β-ara-A; subsequently the acid-soluble fraction was extracted. The amount of radioactivity was determined to be 7.2×10^4 dpm (= 1.5 pmoles with respect to β-ara-A) per 10^7 cells. This fraction was analysed first by ion-exchange chromatography (as described in Materials and Methods). Over 95 per cent of the radioactive material was recovered by elution with 0.1 M NH_4HCO_3, which indicates that (if at all) only minute amounts of β-ara-A could have been phosphorylated intracellularly. For further identification the chromatographed fraction was analysed on thin-layer plates (Fig. 2). The distribution of the radioactivity showed only two peaks, first a peak comigrating with β-ara-Hx (27.2%) and a second one with β-ara-A (63.6%). No pronounced peak of radioactivity was determined at the R_f value of ara AMP ($R_f = 0.0$). These results indicate that β-ara-A is taken up by yeast cells, but is not phosphorylated; however, it was found that β-ara-A is intracellularly metabolized to β-ara-Hx.

Specificity of isolated adenosine kinase. In this

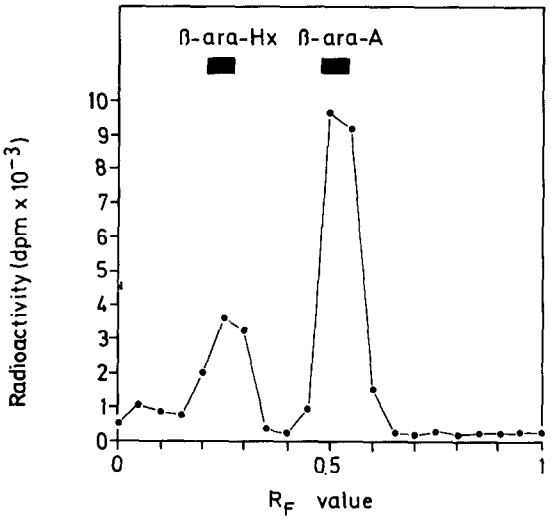

Fig. 2. Characterization of the product formed in yeast cells after incubation with [^{3}H]-β-ara-A. After harvesting the cells were extracted with methanol; the methanol soluble fraction was passed through a QAE-25 Sephadex column; the fraction obtained by elution with 0.1 M NH_4HCO_3 was collected (= 3.5×10^5 dpm) and evaporated to dryness in a rotary evaporator. The residue was dissolved in 200 μl distilled water; 20 μl aliquot was chromatographed on a thin layer plate. The radioactivity of 0.5×1.0 cm spots was determined. The bars mark the R_f values of authentic compounds.

Table 2. Substrate specificity of adenosine kinase from yeast

Nucleoside	K_m (μM)	V_{max} (nmoles NMP/min)
Adenosine	27.48 ± 2.1	84.9
9-β-D-Arabinofuranosyladenine	3800.0 ± 240.0	7.9
9-α-D-Arabinofuranosyladenine	446.4 ± 30.9	18.9
9-β-D-Arabinofuranosyl-6-mercaptopurine	187.7 ± 14.3	0.9
Coformycin	1921.0 ± 147.0	1.8

series of experiments the substrate specificity of adenosine kinase from yeast to eight aranucleosides and three adenosine deaminase inhibitors was determined. Among these compounds ara-U, ara-C, ara-T, ara-Hx, ara-G, deoxycoformycin and iso-coformycin were found not to serve as phosphate acceptor in the adenosine kinase reactions. As summarized in Table 2, only α-ara-A, β-ara-A, ara-6MP and coformycin were phosphorylated by the adenosine kinase. However, compared to the natural substrate adenosine, these four analogues were converted to the corresponding nucleotides with an extremely low reaction velocity. Furthermore, it has been determined whether ara-A inhibits the phosphorylation of adenosine *in vitro*. In kinetic studies it was found that both α-ara-A and β-ara-A inhibit phosphorylation of adenosine in an uncompetitive way. The kinetic constants were determined as follows: control reaction with adenosine in the absence of the analogues $K_m = 27.5 \pm 2.1$ μM, $V_{max} = 84.9$ nmoles NMP/min; adenosine in combination with α-ara-A $K_p = 15.9 \pm 2.0$ μM, $V_p = 45.5$ nmoles NMP/min; adenosine in combination with β-ara-A $K_p = 11.4 \pm 1.1$ μM, $V_p = 32.3$ nmoles NMP/min. The inhibition turned out to be fully uncompetitive because K_m and V_{max} decrease to the same extent; the ratio of K_m/V_{max} in the control reaction was 0.324, K_p/V_p in the β-ara-A inhibited reaction was 0.353 and K_p/V_p in the α-ara-A inhibited reaction was 0.349. The degree of inhibition of the enzymatic adenosine phosphorylation by β-ara-A and α-ara-A is low; the K_i for β-ara-A was determined to be 155 μM and the one for α-ara-A 230 μM.

DISCUSSION

It has been reported that β-ara-A is a strong inhibitor of growth of mammalian cells, bacteria and some viruses [6, 7]. Therefore the results presented in this report which demonstrate that β-ara-A is inactive in the yeast system came unexpectedly, especially after finding that this nucleoside analogue is taken up by yeast cells. The underlying biochemical mechanism of the resistance of yeasts to β-ara-A has been elucidated. It was determined that this nucleoside is not phosphorylated intracellularly to ara-AMP and consequently not to ara-ATP, which, as has been demonstrated, is the ultimate active metabolite both on the DNA polymerase [22, 17] and on the ribonucleotide reductase level [23] in mammalian systems. In animal cells β-ara-A is rapidly phosphorylated to ara-ATP [16]. It is supposed that the phosphorylation of β-ara-A is effected by a distinct deoxyadenosine kinase because purified adenosine kinase isolated from either HEp-2 human tumor cells [24] or rabbit liver [19] does not carry out the conversion from β-ara-A to ara-AMP at significant rates. In the bacterial system (*Escherichia coli*) β-ara-A exerts a killing effect [25]. Although it was found that DNA synthesis is inhibited in *E. coli* after treatment with β-ara-A [25], it is not yet known whether the ultimate active metabolite is the nucleoside or the converted nucleotide. Published data report that ara-ATP does not inhibit *E. coli* DNA polymerase I [22] whereas this nucleotide strongly reduces the activity of adenyl cyclase, purified from *E. coli* K12 [26]. In mammalian tumor cells, both α-ara-A and coformycin were found to be phosphorylated up to the triphosphate level [27, 28].

In the present studies with the isolated adenosine kinase from yeast it was proven that β-ara-A is a poor substrate for this enzyme; the Michaelis constant for β-ara-A is 140-fold higher and the maximum reaction velocity 11-fold lower compared to corresponding kinetic constants of adenosine. In addition, eight further aranucleosides and three deaminase inhibitors (tetrahydroimidazo-diazepin derivatives) that potentiate the β-ara-A inhibitory effect in mammalian cells [29] have been tested and found to be poor substrates for the yeast adenosine kinase as well. This observation supports the assumption that the specificity of the yeast enzyme for nucleosides is rather limited [18]. To clarify whether ara-A acts as an inhibitor for this enzyme reaction, kinetic experiments with adenosine in the presence of the analogues were performed. Both β-ara-A and α-ara-A were found to inhibit enzymatic adenosine phosphorylation in an uncompetitive way. However, because of the high inhibition constants (K_i for β-ara-A: 155 μM and for α-ara-A: 230 μM), these two adenosine analogues must be considered as weak inhibitors.

It is well known that some nucleotides are slowly transported across cell membranes of mammalian cells [30] and more rapidly across cell membranes of yeast [3, 4]. With this knowledge in mind, ara-AMP was added to the *Saccharomyces* cultures; growth experiments revealed that this 5'-monophosphate is a potent inhibitor of yeast cell proliferation as concluded from the low ED_{50} concentration of 8.3 μM. Three indirect pieces of evidence are available to indicate that ara-AMP penetrates as an entire molecule through yeast cell membranes and is intracellularly phosphorylated to ara-ATP: first, the ara-AMP-caused inhibitory effect can be abolished by coincubation with deoxyadenosine; second, ara-AMP causes an inhibition of DNA synthesis in yeast cells which is analogous to the findings in other biological systems [6, 7], probably due to an inhibition of the DNA polymerase by ara-ATP; and

third, the possible ara-AMP degradation compounds adenosine, adenine, α-arabinose and β-ara-A (all these compounds cross the cell membrane of yeasts; Refs. 31, 12, 32 and this paper) were found to cause no inhibition of cell growth.

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