GROWTH INHIBITION OF *Escherichia coli* B BY NUCLEOSIDE ANALOGS*

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The inhibition of growth of *Escherichia coli* B by series of 6-azaanalogs of pyrimidine nucleosides, their precursors, and analogs of cancerostatic agents 5-fluorouracil and arabinosylcytosine is reported. A very high antibacterial activity is observed with most of the 5-fluorouracil nucleosides where a cleavage (30%) to 5-fluorouracil is observed at the most active compounds (5-fluorouridine, 5-fluoro-2'-deoxyuridine). Arabinosylcytosine and its derivatives express very low activity.

For more than 20 years[1,2] a continuous attention has been focused in our laboratories to the syntheses of different types of nucleoside analogs as an effort to find out a biologically active compound, a potent new antimetabolite of nucleic acids. A test for growth inhibition of *E. coli* B utilized for screening of their biological activity was shortly recognized quite unsatisfactory for detection of their cancerostatic activity. It was used exceptionally only, even if it was known that the biological activity of the important antileukemic agent, arabinosylcytosine, was firstly discovered just by *E. coli* test[3] (Table I). When other test methods *in vitro*, fundamentally more efficient, became available for screening of new compounds, in particular a test for inhibitory effect *vs* DNA and RNA synthesis[4–8], *vs* growth of L1210 (ref.[6]) and HSV *1* (ref.[6,8]), the results were compared and a proposal for a primary screening of antitumor activity was postulated[7,8]. The present paper summarizes the results on inhibition of growth of *E. coli* B (Table II).

A series of analogs[2,9–13] derived from pyrimidine antimetabolites, 6-azauridine and/or 6-azacytidine, have been studied at first, in order to examine the influence of a secondary isosteric change on the activity of the original biologically active molecule. The unsubstituted 6-azauracil[14] was found to be by orders more active than its 1-ribosyl derivative[2,9,10]. The bacteriostatic activity of 6-azauridine is almost negligible while it partially retains some neurotoxicity of 6-azauracil due to a partial splitting to the latter one[15]. An effort to replace the labile 1-ribosyl

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TABLE I
Inhibition of growth of *E. coli* B

Compound	Inhibition %	Concentration[a] μg/ml
By pyrimidine nucleoside analogs		
6-Azauracil[14]	90	10
1-Methyl-6-azauracil[14]	0	1 000
6-Azauridine[9,10]	80	1 000
5′-Mesyl-6-azauridine[38]	34	100
O^2,2′-Cyclo-6-azauridine[12,13]	0	1 000
6-Azacytidine[39,40,11]	91	1 000
Arabinosyl-6-azacytosine[12]	78	100
Arabinosyl-6-azaisocytosine[13]	84	1 000
4-Thio-6-azauracil[17]	23	100
4-Thio-6-azauridine[11]	14	100
2′,3′-Isopropylidene-4-thio-6-azauridine[11]	64	100
5′-Acetyl-2′,3′-isopropylidene-4-thio-6-azauridine[11]	67	100
Arabinosyluracil[19]	27	100
O^2,2′-Cyclouridine[19]	0	1 000
Arabinosylisocytosine[13]	26	1 000
Arabinosyl-4-thiouracil[19]	26	1 000
5-Cyanouracil[41]	0	1 000
5-Cyanouridine[41]	31	1 000
5-Azacytosine[31]	64	100
5-Azacytidine[32]	94	10
5-Aza-2′-deoxycytidine[33]	37	10
By acyl cyanide semicarbazone and their cyclisation products		
Acetyl cyanide semicarbazone[21]	100	1 000
Acetyl cyanide thiosemicarbazone[21]	100	1 000
Acetyl cyanide S-methylisothiosemicarbazone[21]	100	1 000
Benzoyl cyanide semicarbazone[21]	26	100
5-Phenyl-6-azacytosine[21]	100	1 000
5-Methyl-6-azacytosine[21]	16	1 000
5-Methyl-2-thio-6-azacytosine[21]	0	1 000
5-Methyl-2-methylthio-5-azacytosine[21]	72	1 000
By 5-fluorouracil derivatives		
5-Fluorouracil	56	0·01
5-Fluorouridine[10]	97	1
5-Fluoro-2′-deoxyuridine[25]	76	0·2

[a] Minimum concentration where the activity is retained.

TABLE I
(*Continued*)

Compound	Inhibition %	Concentration[a] μg/ml
Florafur	95	100
2',5'-Dideoxy-5-fluorouridine[4]	97	1
5'-Deoxy-5-fluorouridine[24]	97	1
5'-Chloro-5-fluorouridine[24]	67	10
5'-Chloro-O[2],2'-cyclo-5-fluorouridine[4]	15	100
2',5'-Dichloro-5-fluorouridine[4]	0	100
5'-Chloro-5-fluoro-2'-deoxyuridine[4]	22	1
2'-Bromo-5'-chloro-5-fluorouridine[4]	17	100
Arabinosyl-5-fluorouracil	24	100
5'-Deoxyarabinosyl-5-fluorouracil[4]	0	100
By arabinosylcytosine derivatives		
Arabinosylcytosine[26,27,19]	85	1 000
Cyclocytidine hydrochloride[26]	19	1 000
Tri-O-acetylarabinosylcytosine[28]	39	1 000
N-Acetylarabinosylcytosine[42] (*cf.*[43])	74	1 000
5'-Chloroarabinosylcytosine[29]	0	1 000
5-Carboxyarabinosylcytosine[30]	0	1 000
5-Carboxycytidine[30]	16	1 000
5-Carboxycytosine[30]	23	1 000
5'-Chlorocytidine[29]	0	1 000
5'-Deoxycytidine[29]	0	1 000
5'-Chloroadenosine[29]	0	1 000
5'-Deoxyadenosine[29]	0	1 000

substituent by a stable 1-alkyl group completely removes the biological activity and thus the 1-alkyl derivatives are not able to substitute the low toxic 6-azauridine. The bacteriostatic activity and toxicity of 6-azauracil, by orders higher than those of 6-azauridine, are surprising from the point that the 5'-monophosphate ester of 6-azauridine is proposed[16] the proper biologically active form of the 6-azauracil derivatives. Second and further isosteric and/or configurational changes introduced into the molecule of 6-azauridine and/or 6-azacytidine did not enhance the biological activity of the parent molecule. Only at the 4-thio derivatives of 6-azauracil[17] and 6-azauridine[11], and at arabinosyl-6-azacytosine[12] a mildly enhanced activity was observed. On the other hand, the very reactive and promising compound[13,18], O[2],2'-cyclo-6-azauridine, even did not reach the activity of the parent compound and did not, equally as arabinosyl-4-thiouracil[19], meet our expectation.

The precursors in the syntheses of substituted 6-azauracils[14,20] and 6-azacytosines[21] were also measured. In the series of acyl cyanide semicarbazones and thiosemicarbazones[21], these precursors were always found more active than the products of cyclisation, *i.e.*, the corresponding 5-substituted 6-azacytosines; the benzoylcyanide derivative possessing the highest activity. In the series of semicarbazones of glyoxylic acid derivatives[14,20], the corresponding acids were compared with their esters, amides and corresponding cyclic products (*i.e.*, substituted 6-azauracils). The amides possess the highest activity even in comparison with the corresponding 6-azauracils, except for the unsubstituted 6-azauracil itself.

Out of the 5-fluorouracil analogs, the 5-fluorouracil itself expresses far the highest activity, reaching the 60% inhibition of growth of *E. coli* at a minimum concentration $1 . 10^{-2}$ μg/ml. The 5-fluoro-2'-deoxyuridine 5'-monophosphate is proposed to be the proper active form[22] (*cf.*[23]) for the 5-fluorouracil and for its nucleoside derivatives. Therefore, the 5'-deoxy derivatives of the ribo[24] and 2'-deoxyribo[4] series, which are unable to give the 5'-monophosphate ester, were compared with 5-fluorouracil and its ribo[10] and deoxyribo[25] nucleosides. Preliminary tests indicated a strong bacteriotoxicity of all these 5-fluoro derivatives, expressing an identical inhibitory effect (90%) at a concentration of 10^{-6} g/ml. A more detailed study was performed with a larger series of 5-fluorouracil derivatives. Besides of 5-fluorouracil, the 5-fluoro-2'-deoxyuridine was observed the second strongest inhibitor. On the other site, the 2',5'-dihalogeno derivatives[4], 5'-deoxyarabinosyl[4] and arabinosyl derivative itself as well as 5'-chloro-O^2,2'-cyclo-5-fluoro-uridine[4] belong to the lowest inhibitors of *E. coli*. The dependence of the biological activity on potential cleavage of nucleoside bond was studied with some 5-fluorouracil nucleosides (Table III). The liberation of the extremely bacteriostatic 5-fluorouracil should then be responsible for the

TABLE II

Inhibition (%) of *E. coli* B by semicarbazones of glyoxylic acid derivatives (standard concentration 1 mg/ml)

Compound	Ester	Acid	Amide	Cyclization product
Methyl glyoxylate (*E*)-semicarbazone[14]	0	78	74	88[a]
Methyl glyoxylate (*Z*)-semicarbazone[14]	41[b]	—	—	88[a]
Methyl glyoxylate 2-methylsemicarbazone[14]	0	2	18	0
Methyl glyoxylate 2-benzylsemicarbazone[14]	—	37	—	48
Methyl glyoxylate 4-phenylsemicarbazone[14]	—	—	—	61
Methyl glycoxylate 2-ribofuranosylsemicarbazone[9]	—	—	89	72

[a] Concentration 0·01 mg/ml; [b] concentration 0·1 mg/ml.

high activity of the parent compounds. Even if not an unambiguous correlation was found, some relation between the biological activity and the cleavage of nucleoside bond could be observed. The most active nucleosides (fluorodeoxyuridine, fluorouridine) are cleaved approximately by 1/3 while the arabinosyl-5-fluorouracil and 5'-chloro-5-fluorouridine[24] are not cleaved at all.

No surprising results were observed in the series of arabinosylcytosine[26,27,19] which possesses a very low inhibitory activity *vs E. coli* at the basic concentration 1 mg/ml, only. All its derivatives[28–30] possess a lower activity then the parent arabinosylcytosine. The 5'-deoxy derivatives of cytidine and adenosine[29] express no activity at the basic concentration 1 mg/ml. From the 5-azaanalogs[31–33], the riboside, 5-azacytidine[32], is the strongest inhibitor while the nucleobase, 5-azacytosine[31], the lowest one at the concentration 10 μg/ml.

The most potent cancerostatic nucleoside analogs, arabinosylcytosine and cyclocytidine, possess an almost negligible inhibitory effect *vs* growth of *E. coli*. On the other hand, 6-azauracil is one of the strongest inhibitors of *E. coli* but does not inhibit NA synthesis at all[7,8] and has no use in cancer treatment. Similarly, most of the 5-fluorouracil derivatives exhibit an extreme activity *vs E. coli*, even those which do not inhibit the NA synthesis[4,25].

In conclusion, it should be accepted that there is a negligible relation between the activity *vs* growth of *E. coli* and the cancerostatic activity as presented at the studied compounds. The value of the system using *E. coli* consists in the study of mechanism of action of the active compounds and of their metabolims by utilization of the complex enzymatic system of the wild strain of *E. coli* B.

EXPERIMENTAL

The bacteriostatic activity was tested by the strain of *Escherichia coli* B which is anaerogenic, non-motile, lysine and ornithine-decarboxylase and arginin-dihydrolase negative lactose fermenting

TABLE III

Cleavage of 5-fluorouracil derivatives to 5-fluorouracil by *E. coli* B

Compound	Inhibition %	Concentration μg/ml	Cleavage %
5-Fluoro-2'-deoxyuridine[25]	76	0·2	36
5-Fluorouridine[10]	90	1	31
5'-Deoxy-5-fluorouridine[24]	90	1	0
5'-Chloro-5-fluorouridine[24]	67	10	0
Ftorafur	66	100	5
Arabinosyl-5-fluorouracil	25	100	0

and sucrose nonfermenting; S → R colonial variation. The strain expresses a sensitivity (standard technique) to amoxicillin, amoxicillin with clavulanic acid, carbenicillin. cefazolin, cefotaxim, cefoxitin, chloramphenicol, colistin, doxycycline, gentamicin, nalidixic acid, netilmicin, polymyxin B, sisomicin, ticarcillin; a moderate sensitivity to amikacin, ampicillin, cefalotin, kanamycin, neomycin, nitrofurans, tetracycline; and a resistance to azlocillin, co-trimozale, rifampicin streptomycin.

Escherichia coli B was grown on a synthetic medium[34] (*cf.* ref.[35]) containing 0,3% of glucose. The cultivation, after addition of single test substances, was stationary incubated at 37°C for 16 h. The growth of cultivation was evaluated turbidimetrically on a Spectrophotomer Spekol 10 (Carl Zeiss, Jena) at 575 nm. The test substances were synthesized as indicated by references.

The samples (1 ml) selected for determination of 5-fluorouracil derivatives and their metabolism were precipitated by addition of 96% ethanol (2 ml), when the incubation was finished. The precipitate was removed by centrifugation (2·000 rpm, 10 min). The supernatant (1 µl), containing the substrate and its metabolites, was directly used for quantitative evaluation by liquid chromatography on silica gel. For HPLC the equipment described recently[36,37] was used. The normal-phase chromatography was performed on LiChrosorb 100 (Merck, Darmstadt) by mobile phase systems as indicated in Table IV. Stainless steel columns, 250 mm long, 4·2 mm inner diameter, inlet pressure 7,2 MPa, and flow rate 1 ml/min were used. The detection was performed spectrophotometrically at 254 nm.

TABLE IV

Capacity factors k of 5-fluorouracil derivatives on silica gel. Column: packing, LiChrosorb SI 100, 5 µm (250 mm × 4·2 mm ID). Mobile phase systems (parts by volume): A 78 dichloromethane, 20 methanol, 2 ammonium formate (0·5 mol l^{-1}, pH 3·0); B 80 dichloromethane, 18 methanol, 2 ammonium formate (0·5 mol l^{-1}, pH 3·0); C 78·5 dichloromethane, 19 methanol, 2·5 ammonium formate (0·5 mol l^{-1}, pH 3·0)

Compound	k		
	A	B	C
5-Fluorouracil	0·92	1·30	1·19
5-Fluorouridine[10]	2·28	3·89	2·99
5-Fluoro-2′-deoxyuridine[25]	1·29	1·97	1·66
Ftorafur	0·20	0·16	0·15
5′-Deoxy-5-fluorouridine[24]	0·92	1·30	1·07
5′-Chloro-5-fluorouridine[24]	0·82	1·11	0·90
5′-Chloro-O^2,2′-cyclo-5-fluorouridine[4]	0·95	1·10	0·94
Arabinosyl-5-fluorouracil	2·03	3·47	2·77
5′-Deoxyarabinosyl-5-fluorouracil[4]	1·03	1·46	1·18
2′,5′-Dichloro-5-fluorouridine[4]	0·37	0·40	0·35
2′-Bromo-5′-chloro-5-fluorouridine[4]	0·34	0·37	0·33
5′-Chloro-5-fluoro-2′-deoxyuridine[4]	0·46	0·54	0·47
2′,5′-Dideoxy-5-fluorouridine[4]	0·52	0·63	0·56

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