An Improved Tissue Culture Assay

II. Cytotoxicity Studies with Antibiotics, Chemicals, and Solvents*

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In the preceding publication of this series (10), a simplified tissue culture assay was described. The cytotoxic activity of various nucleic acid antagonists, antibiotics, amino acid and vitamin antagonists, respiratory poisons, miscellaneous chemicals, solvents, heavy metals, detergents, and fermentation beers were determined with the new assay procedure and are reported in this publication.

MATERIALS AND METHODS

Details of assay methodology (with the use of Eagle's KB strain of human carcinoma cells) were described in the preceding publication (10). Test samples (sterilization unnecessary) were weighed into sterile bottles and dissolved in water (sterile), dimethylformamide (nonsterile), or ethanol (nonsterile). Solvents were diluted in sterile water to a final concentration of less than 1000 μg/ml. For preliminary screening, test compounds were assayed at 500, 100, 20, 4, and 0.8 μg/ml with one assay tube per concentration. Agents with an ID_{50} (dose for 50 per cent inhibition) greater than 100 were not reassayed. Interesting agents (ID_{50} less than 10 μg/ml) were reassayed at four or five concentrations, with two tubes per level. Stock solutions of test agents were stored at −10° C. for a maximum of 3 weeks for retest. If the agent was suspected or known to be unstable, a fresh test solution was prepared for each assay.

TABLE 1

ACTIVITIES OF ANTAGONISTS WHICH AFFECT NUCLEOTIDE OR NUCLEIC ACID METABOLISM

AGENT	NSC NO.*	ID_{50}, μG/ML		NO. EXPERIMENTS
		Median	Range	
Amethopterin	740	0.01	<0.003–0.015†	8
Aminopterin	739	0.003	$0.6–6\times10^{-3}$	5
8-Azaguanine	749	0.035	0.02–0.065	5
Azaserine	742	2.0	1.8–3.6	5
5-Bromouracil	19940	>500	>500	3
5-Bromouridine		460	380–460	3
Colchicine	757	0.002	0.002	5
5-Fluorouracil	19893	0.8	0.52–1.1	6
5-Fluorodeoxyuridine	27640	0.008	0007–0.015	4
5-Fluorouridine		0.008	<0.003–0.012‡	9
6-Mercaptopurine	755	0.07	0.05–>0.2§	12
Purine	753	1.0	0.7–1.2	3
Puromycin aminonucleoside	3056	2.5	2–3	2
6-Thioguanine	752	0.025	0.020–0.029	2

* Cancer Chemotherapy National Service Center number.

† Range was 0.008–0.015 in seven of eight experiments.

‡ Range was 0.004–0.012 in eight of nine experiments.

§ Range was 0.05–0.14 in nine of twelve experiments.

Nucleic acid nucleotide inhibitors.—The inhibitory activities of several antagonists which affect nucleotide or nucleic acid metabolism are shown in Table 1.

Most of these antagonists are very potent inhibitors of

* This work was supported by Contract No. SA-43-ph-1933, Cancer Chemotherapy National Service Center, National Institutes of Health.

Received for publication March 9, 1959.

TABLE 2

ACTIVITIES OF ANTIBIOTICS* VS. KB CELLS

AGENT	NSC NO.	ID_{50}, μG/ML		No. EXPERIMENTS
		Median	Range	
Actinomycin D	3053	0.00006	$1.5–30\times10^{-5}$	4
Althiomycin		>100	70–300	3
Amicetin	5340	7	6–9	3
Amphomycin		>250	>250	2
Carbomycin		20	12–20	3
Carzinophilin	20088	0.005	0.0004–>0.01	5
Catenulin		>500	>500	3
Celesticetin		230	180–280	3
Chartreusin	5159	1.3	1–2	3
Chloramphenicol	3069	25	12–53	5
Chlortetracycline·HCl	13252	50	43–62	3
Filipin	3364	1.5	0.95–1.7	3
Fungichromin		2.7	2.7–2.8	2
Kanamycin		>500	>500	2
Leucomycin		10	9.5–10	3
Mitomycin C	26980	0.025	0.014–>0.04†	5
Neomycin		>500	>500	2
Nitrofurazone	2100	35	35–38	2
Pentamycin		2		1
Pluramycin		0.003	0.001–0.005	2
Proactinomycin		28	20–32	2
Sarkomycin	14347	>100	>24–130	2
Streptolin-streptothricin		300	210–390	2
Tetracycline·HCl		33	31–61	3
Tubercidin		0.02	<0.01–0.035	3
Xanthomycin		0.005	0.003–0.007	2

* The material assayed was the purest preparation available. In many cases, the preparations were noncrystalline.

† Range was 0.014–0.025 in four of five experiments.

TABLE 3

ACTIVITIES OF VARIOUS AMINO ACID AND VITAMIN ANALOGS VS. KB CELLS

AGENT	NSC No.	ID_{50}, μG/ML		No. EXPERIMENTS
		Median	Range	
Amino acid analogs:				
L-3-Aminotyrosine		230		1
DL-Ethionine	751	280		1
Methionine sulfoxide	3084	>500		1
DL-α-Methylglutamic acid		>500		1
DL-β-Phenyllactic acid	2627	400		1
β-2-Thienylalanine	754	>500		1
β-2-Thienylserine		>500		1
Vitamin analogs:				
Acetylpyridine	761	>500		1
6-Aminonicotinamide	21206	5	3.5–6.5	3
Deoxypyridoxine	3063	15	13–20	3
DL-Desthiobiotin	3085	>500		1
Neo-pyrithiamine		>500		1
Oxythiamine		>500		1
Pantoyl taurine	3086	>500		2
10-(2-Acetoxyethyl)-7,8-dimethyl-isoalloxazine	3064	65	55–75	2

KB cells *in vitro* under these test conditions (Table 1). 5-Bromouracil and bromouridine are notable exceptions. Rich *et al.* (9) have reported that fluorouracil completely inhibited the growth of H.Ep. #1 cells in tissue culture at 1 μg/ml and that the nucleosides inhibited at 0.01 μg/ml. The data reported above corroborate their findings completely.

Antibiotics.—Table 2 shows the cytotoxicities of various antibiotics in the KB assay. The activities of cycloheximide[1] and the streptovitacins were reported elsewhere (10).

These data (Table 2) show a wide spectrum of cytotoxicities ranging from $ID_{50} = 6 \times 10^{-5}$ μg/ml for actinomycin D to greater than 500 μg/ml for several antibiotics and illustrate the wide range of activities which can be expected in fermentation beers. Actinomycin D is the most cytotoxic agent tested thus far in these laboratories.

Amino acid and vitamin analogs.—Table 3 shows the activities of various amino acid and vitamin analogs.

None of the amino acid analogs tested was highly toxic to KB cells under these conditions of test. Of the vitamin antagonists tested, only 6-aminonicotinamide and deoxypyridoxine showed interesting activities in the KB assay.

Oxidative, glycolytic, and respiratory inhibitors.—Table 4 summarizes the cytotoxic activities of several agents known

[1] β-[-2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide.

TABLE 4

ACTIVITIES OF METABOLIC INHIBITORS VS. KB CELLS

AGENT	NSC NO.	ID_{50}, μG/ML		No. EXPERIMENTS
		Median	Range	
Amytal (sodium)		>300		1
2,4-Dinitrophenol	1532	35	32–38	3
Fluoroacetic acid (sodium salt)		>500	>500	2
Iodoacetic acid	2125	0.4	0.35–>0.8*	5
Malonic acid		410	320–500	2
Potassium fluoride		220	200–240	2
Sodium arsenate(·7 H_2O)		33	21–33	3
Sodium azide		6	2.5–30†	6
Sodium cyanide		10	<10–20	4

* Range was 0.35–0.6 in four of five experiments.

† Range was 2.5–7 in five of six experiments.

TABLE 5

ACTIVITIES OF MISCELLANEOUS CHEMICALS VS. KB CELLS

COMPOUND	NSC NO.	ID_{50}, μG/ML		No. EXPERIMENTS
		Median	Range	
Acetic acid		>1000		1
Aconitic acid	7616	>1000		1
Amidinourea sulfate	3123	350		1
Ammonium chloride		> 500		1
1H-Benzotriazole	3058	150	120–190	2
2-Benzyl-2-thiopseudourea·HCl	3206	7	6–8	2
p-Biguanidobenzamide	5201	160		1
Carbamic acid, ethyl ester	746	> 500		1
Citric acid	30279	>1000		1
Daraprim	3061	2.5	<2.5–10	3
2-(2,4-Dichlorophenoxy)ethanol	423	45		1
2-Ethylamino-1,3,4-thiadiazole	4730	> 500		1
Fumaric acid	2752	>1000		1
Gluconic acid		>1000		1
17-Hydoxycorticosterone, 21-acetate	741	240		1
Malic acid	9232; 25941	>1000		1
Nitrogen mustard	762	0.2	0.15–0.2	2
Pyronine B	3093	0.3	0.2–0.4	2
Pyrogallol	5035	8	6–12	3
Quinine·HBR	12865	45	30–60	2
2-(*p*-Dimethylaminostyryl)-1-methylquinolinium iodide	4238	0.2	0.1–0.5	3
4-(*p*-Dimethylaminostyryl)quinoline	4236	5	4–15	4
Succinic acid	25949	>1000		1
Strophanthin K	4320	0.02	0.006–0.02	3
Urea		> 500		1

to inhibit metabolism in the glycolytic Krebs cycle, or respiratory pathways.

The lack of marked inhibition by most of the agents shown in Table 4 is surprising. Lack of permeability can hardly be assumed for such a variety of chemical structures. It is interesting that fluoroacetic acid was nontoxic to KB cells in this test system when iodoacetic acid was highly inhibitory. Acetate has been shown to penetrate mammalian cells in tissue culture (2), and fluoroacetate probably enters also. The Krebs cycle is operative in mammalian (HeLa) cells *in vitro* (1), and fluoroacetate would be expected to be quite inhibitory, since it is so toxic *in vivo* (3). Amytal has been reported to inhibit hydrogen transport at the flavin level (5, 6), but it was essentially inactive in this system. The cytochrome poisons (dinitrophenol, azide, arsenate, and cyanide) were mildly inhibitory in this test system.

Miscellaneous organic chemicals.—A series of chemicals was submitted under blind label by the Cancer Chemotherapy National Service Center (CCNSC). These agents and several miscellaneous metabolites were tested by the routine screening procedure, and the results are shown in Table 5.

TABLE 6

ACTIVITIES OF ORGANIC SOLVENTS VS. KB CELLS

Solvent	NSC no.	ID_{50}, μg/ml	No. experiments
Acetone		>5000	3
n-Butanol		>1000	1
t-Butanol		>1000	1
Dimethylacetamide	3138	>5000	1
Dimethylformamide	5356	>5000	3
Dioxane (1, 4)	8728	200–800	1
Ethanol		>5000	3
Methanol		>5000	2
n-Propanol	30300	>1000	1
i-Propanol		5000	1

In addition to the chemicals shown in Table 5, the CCNSC also submitted aminopterin, purine, thioguanine, colchicine, puromycin aminonucleoside, nitrofurazone, strophanthin K, pantoyl taurine, sarkomycin, mitomycin C, carzinophilin, and sodium azide, the activities of which are reported in other tables.

Solvents.—Since water-insoluble compounds must be dissolved in organic solvents for tissue culture testing, the inhibitory activities of several solvents to KB cells were determined and are shown in Table 6.

The lack of cytotoxicity with these solvents was unexpected but reproducible. Ethanol and dimethylformamide were chosen to dissolve water-insoluble compounds for tissue culture testing, and the solutions were diluted to give a final solvent concentration of less than 1000 μg/ml. Appropriate solvent blanks were always included.

Mercury, copper, and chromium.—Mercuric and dichromate ions are inhibitory to many biological systems. In the KB assay, the median ID_{50} for mercuric chloride was 10 μg/ml (four experiments) and for sodium dichromate·$2H_2O$ it was 0.2 μg/ml (four experiments). Copper sulfate·$5H_2O$ had a median ID_{50} of 35 μg/ml (four experiments). Dichromate was substantially more toxic than mercuric or cupric ions.

Detergents and trypsin.—Great care is generally exercised in removing traces of detergent from tissue culture glassware. Furthermore, "residual detergent" is often cited as the cause when cells suddenly fail to grow normally. The cytotoxicities of several commonly used detergents were investigated in the KB assay and are reported in Table 7. Trypsin was included in this series to compare its activity with that of versene, since both are used to remove cells from glass (7, 11).

It is interesting that most of the detergents tested were not inhibitory to KB cells at 100 μg/ml, although they were added before the cells had attached to glass. While it is not suggested that diligent rinsing of tissue culture glassware be abandoned, it is felt that inhibition of KB cells in serum-containing medium will not be caused by residual detergent if common laboratory rinsing is exercised.

Fermentation beers.—Various clarified beers and extracts have been tested against KB cells in tissue culture. These fermentation products exhibited ID_{50} values ranging from less than 5×10^{-4} μg/ml to greater than 500 μg/ml, as was expected. Fractionation studies have begun on some fermentation products.

Comparison with data of Eagle and Foley.—The qualitative agreement between data obtained in these laboratories and those reported by Eagle and Foley (4) is shown in Table 8.

Since the data of Eagle and Foley are available in concentration ranges of tenfold, it is difficult to make a quantitative comparison. With the exception of actinomycin D and 4-(*p*-dimethylaminostyryl)-quinoline, however, the series of compounds shown in Table 8 would have been placed in approximately the same relative order of activity by either test method.

TABLE 7

ACTIVITIES OF DETERGENTS AND TRYPSIN VS. KB CELLS

Detergents	ID_{50}, μg/ml		No. experiments
	Median	Range	
Alconox	>500	>100 & >500	2
Calgon	>500	>100 & >500	2
Hemosol	>500	>100 & >500	2
Microsolv	>100	>100	2
Sodium lauryl sulfate	25	20 & 30	2
Sodium metasilicate	>500	>100 & >500	2
Trypsin	>100	>100	3
Versene (Na)	>100	>100	3

DISCUSSION

The results presented in this paper show that many agents commonly predicted to be "toxic" to mammalian cells in tissue culture were only moderately inhibitory or noninhibitory (ID_{50} greater than 100 μg/ml) to KB cells in this assay system. These agents include vitamin and amino acid analogs, detergents, certain metabolic inhibitors including dinitrophenol, fluoroacetate and malonate, solvents, and some antibiotics and fermentation beers. Mercuric chloride, 6-aminonicotinamide, arsenate, deoxypyridoxine, tetracycline, chloramphenicol, and certain beers and chemicals showed intermediate activities in tissue culture (ID_{50} =

5–50 μg/ml). Dichromate, iodoacetate, most of the nucleic acid antagonists, several antibiotics, and some antibiotic beers were highly inhibitory to KB cells *in vitro* ($ID_{50} < 1$ μg/ml). Actinomycin D was the most cytotoxic agent tested ($ID_{50} = 6 \times 10^{-5}$ μg/ml). It is entirely possible that the presence of 10 per cent serum in the test medium modifies the cytotoxicities of certain materials. Thus, metal ions (Cu, Hg) might be adsorbed to protein and their toxicities masked. This cannot account for the lack of activity in such diverse chemicals as fluoroacetate, detergents, and solvents, however. In fact, ethanol was found to be essentially nontoxic to chick embryo cells at a level of 1 per cent in both synthetic medium and a medium containing naturally occurring ingredients (8).

Supplementation of the assay medium with lactalbumin hydrolysate resulted in decreased sensitivity and possibly increased reproducibility over the standard method with 6-mercaptopurine. The streptovitacin A assay was more sensitive and reproducible when supplemented with lactalbumin hydrolysate (10).

The question of correlation between antitumor activity in experiment animal tumors *in vivo* and cytotoxicity to mammalian cells *in vitro* is raised regularly regarding the utility of tissue culture in the search for new antitumor agents. As is often the case, many agents which were synthesized to inhibit a specific cofactor or enzymatic reaction are noninhibitory to KB cells in this test system. Other agents, chosen at random or isolated from natural or fermentation sources, are highly inhibitory under the same conditions. How active these cytotoxic compounds might be *in vivo* must be determined by experimentation. The question of correlation with antitumor activity can be answered only after some new agents have been isolated as inhibitors of mammalian cells *in vitro* and tested against a broad spectrum of experimental tumors *in vivo*.

Fermentation and extraction studies can be carried out with the tissue culture assay in a manner analogous to that used in antibacterial systems. As can be seen from the data presented above, inhibition of KB cells in tissue culture under these conditions of test is not a nonspecific phenomenon which can be expected to occur with most products of metabolic origin. Many antibiotic beers and extracts are much more cytotoxic to KB cells *in vitro* than are mercuric, cupric, and dichromate ions, iodoacetate, arsenate, fluoroacetate, and other toxicants.

The kinds of inhibitors which will be isolated from antibiotic beers with the aid of a tissue culture assay remain to be determined. We feel that new compounds, which defy synthesis and defeat rationale, can be isolated from fermentation products with the aid of the tissue culture assay. Many of the common or known inhibitors can be identified and rejected by the judicious use of other *in vitro* tests such as antibacterial and antifungal systems. The agents isolated from such a program certainly merit broad testing in experimental tumors and other biological systems and perhaps, in certain cases, in human neoplasia as well.

TABLE 8

COMPARISON OF CYTOTOXICITY IN TWO TEST SYSTEMS

COMPOUND	NSC NO.	ID_{50}, μG/ML	
		Eagle and Foley (5)	Present assay
Colchicine	25	10^{-2}–10^{-3}	10^{-2}–10^{-3}
Strophanthin K	64	10^{-2}–10^{-3}	10^{-1}–10^{-2}
4-(*p*-Dimethylaminostyryl) quinoline	87	10^{-1}–10^{-2}	10 – 1
Actinomycin D	97	10^{-1}–10^{-2}	10^{-4}–10^{-5}
6-Thioguanine	27	10^{-1}–10^{-2}	10^{-1}–10^{-2}
Cycloheximide	46	10^{-1}–10^{-2}	10^{-1}–10^{-2}
Azaserine	73	1 –10^{-1}	10 – 1
Daraprim	99	1 –10^{-1}	10 – 1
6-Mercaptopurine	105	1 –10^{-1}	10^{-1}–10^{-2}
Purine	128	1 –10^{-1}	10 – 1
Pyronine B	103	1 –10^{-1}	1 –10^{-1}
8-Azaguanine	162	1 –10^{-1}	10^{-1}–10^{-2}
Puromycin aminonucleoside	5	1 –10^{-1}	10 – 1
Deoxypyridoxine·HCl	24	10 – 1	100 –10
Sodium azide	92	10 – 1	10 – 1
2,4-Dinitrophenol	32	10 – 1	100 –10
Nitrofurazone	172	10 – 1	100 –10
DL-Ethionine	114	100 –10	>100
DL-Desthiobiotin	21	100 –10	>100
β-2-Thienylaline	151	>100	>100
Chloramphenicol	89	100 –10	100 –10
Methionine sulfoxide	8	>100	>100

SUMMARY

1. The cytotoxic activity of a variety of chemicals, metabolic antagonists, antibiotics, detergents, solvents, and fermentation beers were determined in a simplified tissue culture assay with the KB strain of human epidermoid carcinoma cells.

2. Nucleic acid antagonists, antibiotics, fermentation beers, and certain chemicals proved to be highly inhibitory ($ID_{50} = 6 \times 10^{-5}$–1.0 μg/ml) to KB cells. Several metabolic antagonists, e.g., arsenate, malonate, fluoride, dinitrophenol, and fluoroacetate, were only slightly active or totally inactive in this test.

3. Methanol, ethanol, dimethylformamide, and other solvents had ID_{50} values greater than 5,000 μg/ml. Dioxane was the most toxic solvent tested, with ID_{50} in the range of 500 μg/ml.

4. Of seven detergents tested, six had ID_{50} values greater than 100 μg/ml. Sodium lauryl sulfate was the most toxic detergent, with ID_{50} = 25 μg/ml.

ACKNOWLEDGMENTS

The skilled technical assistance of Anita Krivis, Marilyn Norman, Lorna Schroeder, and Richard Washburn is gratefully acknowledged.

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