Use of a *Lactobacillus casei* Assay as a Prescreen for Potential Anticancer Agents: An Update Study

THEODORE T. OTANI *x, MARY R. BRILEY *, and RUTH I. GERAN ‡

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Abstract □ Updating an earlier report, 15 known antineoplastic agents, 11 of which are commercially available for treatment of human cancers, were tested for growth-inhibitory activity in a *Lactobacillus casei* system to determine if this microbial system could select them as "active." Although we previously reported that over 160 compounds tested in this assay were inactive, 11 of the 15 known antineoplastic agents tested here were inhibitory. Because of this observation and the advantage that the procedure is rapid and inexpensive, this microbiological assay is recommended for consideration as a prescreen for anticancer agents.

Keyphrases □ *Lactobacillus casei*—anticancer agents □ Anticancer agents—lactobacillus casei

The need for a simple, rapid, and inexpensive method of screening for potential anticancer agents has long been recognized. In 1958, an *ad hoc* committee[1] was organized by the National Cancer Institute Div. of Cancer Treatment to investigate the feasibility of using microbiological systems for such a purpose. As a result of the study, the committee published a comprehensive report in which it described 16 assays and the use of a set of four microbial systems capable of detecting 95% of 89 compounds from among some 200 compounds known at that time to be effective in *in vivo* assay against experimental neoplasms (1). Although certain limitations in the microbiological procedure were recognized, its possible role in the search for new agents was recognized.

In the present study 15 compounds currently being used for the treatment of human malignancies or being developed for clinical use by the National Cancer Institute were tested for growth-inhibitory activity in a *Lactobacillus casei* system.

EXPERIMENTAL

Fifteen anticancer agents[2] currently being used, clinically or in a developmental phase in the cancer treatment program, and soluble in aqueous systems were selected at random (Table I). Twenty milligrams of the test compound was dissolved in 10 mL of triple-distilled (in glass) water. In some cases sufficient amounts of 0.1 M sodium hydroxide were added to bring the test solution to pH 6.0 before bringing to volume. The solutions were sterilized by filtration through filter units[3] attached to a sterile 10-mL plastic syringe[4]. The test solutions were pipetted in graded volumes to contain 0.1, 0.5, and 1.0 mg/mL, final concentration, of the test compound. The volume was made to 2.5 mL by the addition of an appropriate volume of sterile water. The final volume of the assay system after the addition of the medium was 5.0 mL.

The assay procedure is that of Foley *et al.* (1) and is also described in our earlier paper (2).

A riboflavin assay medium[5] supplemented with a minimal quantity of riboflavin[6] to give maximal growth (0.03 μg/mL final concentration) was used. The period of medium autoclaving was monitored carefully to not exceed the 10-min period previously described (1).

The organism used was *Lactobacillus casei* ATCC 7469[7], which was carried on agar[8] as stab cultures, transferred monthly, incubated at 37°C for 24 h, and then stored at 4°C.

The inoculum for the assay was prepared by washing a 24-h broth[9] culture (grown at 37°C) in isotonic saline solution, suspending the bacterial pellet in 10 mL of isotonic saline by centrifugation, and from this making a 1:20 dilution suspension in isotonic saline.

An appropriate amount of the medium for the uninoculated control and the solution blank was removed, and the balance of the medium was inoculated with a sufficient volume of the 1:20 bacterial suspension so that each 5 mL contained 0.06 mL of the inoculum. Then 2.5 mL of the inoculated medium was added to each assay tube, which contained the graded amounts of the test solution. The sterility blank and reagent blank tubes containing the uninoculated medium were each treated with 0.06 mL of the isotonic saline solution that is used in washing the bacteria.

The pH of the assay system containing the highest concentration of the test compound determined in a parallel nonsterile system was within ±0.2 pH units of the control tubes containing an equal volume of water in place of the test solution.

The assay was incubated at 37°C for 19 h, after which growth was terminated by immersing the tubes in boiling water for 10 min. The extent of growth was determined turbidimetrically on a photoelectric colorimeter[10] equipped with a red filter (660 mμ). A positive control, consisting of 6-mercaptopurine at 0.1 mg/mL, a normal growth control, containing no test compound, and a sterility control were included routinely in all assays. An inhibition value of 52–56% by 6-mercaptopurine and a concurrent normal growth of 194–200 turbidity reading in the determination were the criteria of a valid assay. The figures shown in the table are typical values from at least three duplicate determinations.

RESULTS AND DISCUSSION

Fifteen compounds, most of which are currently being used clinically as antineoplastic agents, were tested for growth-inhibitory activity in a *Lactobacillus casei* system. The system was a portion of a set of 16 microbial systems selected by the Subcommittee on Microbiology (1), mentioned earlier, for the purpose of screening chemical compounds for potential antitumor activity. According to the study protocol compounds showing a growth inhibition of ≥50% at a concentration of 1 mg/mL were considered inhibitory.

As seen in Table I, 11 of the 15 compounds tested (73%) were inhibitory in the test system: the extent of inhibition was quite striking. Indeed, 10 of the 11 inhibitory compounds showed essentially complete inhibition at 0.5 mg/mL, and 6 of these showed essentially complete inhibition even at 0.1 mg/mL. The inhibition by phosphonoacetyl-L-aspartic acid (PALA) at 1 mg/mL (54%) is considered to be positive as defined by the protocol, even though the inhibition did not appear to be dose related.

The positive tests obtained from this series of compounds are somewhat lower than the 95% (using four microbial systems) or the 90% (using three microbial systems) described by Foley *et al.* (1). However, it is noteworthy that a single microbial system used here was capable of detecting a majority of the 15 selected antineoplastic compounds presently being used clinically or in the developmental phase of the cancer treatment program. The compounds run concurrently with these compounds and found to be negative in this system (*i.e.*, *N*-benzoyl-L-aspartic acid and *N*-propionyl-L-alanine) were also negative in mammalian *in vivo*

[1] Subcommittee on Microbiology, Screening Panel, Cancer Chemotherapy National Service Center, National Cancer Institute, Public Health Service, Bethesda, MD 20205.

[2] Obtained from Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20205.

[3] Millex-GS 0.22 μm filter units, Millipore Corp., Bedford, MA 01730.

[4] BD Plastipak syringe, Becton, Dickinson and Co., Rutherford, NJ 07070.

[5] Difco Laboratories, Detroit, MI 48232.

[6] Calbiochem, San Diego, CA 92112.

[7] From American Type Culture Collection, Rockville, MD 20852.

[8] Micro Assay Culture Agar, Difco Laboratories, Detroit, MI 48232.

[9] Micro Inoculum Broth, Difco Laboratories, Detroit, MI 48232.

[10] Klett-Summerson.

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Table I—Effect of Some Antineoplastic Agents on the Growth of *L. casei* 7469

Compound	NSC[a] No.	Inhibition, %[b] 0.1 mg/mL[c]	0.5 mg/mL[c]	1.0 mg/mL[c]
Doxorubicin hydrochloride[d]	123127	90	—[e]	—[e]
Daunorubicin[d]	82151	99	—[e]	—[e]
Mitomycin[d]	26980	94	—[e]	—[e]
Streptozocin[d]	85998	100	100	100
Phosphonoacetyl-L-aspartic acid[f]	224131	42	50	54
Azaserine[f]	742	100	100	100
Alanosine[f]	153353	42	90	99
DON[f]	7365	100	100	100
Melphalan[d]	8806	0	0	8
Cyclophosphamide[d]	26271	1	0	2
Thiotepa[d]	6396	28	93	98
Cytarabine[d]	63878	59	97	98
Hydroxyurea[d]	32065	0	0	8
Procarbazine[d]	77213	0	−6[g]	−6[g]
6-Mercaptopurine[d]	755	55	100	100
N-α-Benzoyl-L-aspartic acid[h]	227406	0	0	0
N-Propionyl-L-alanine[h]	270569	2	2	2

[a] National Service Center number. The Cancer Chemotherapy National Service Center was the forerunner of the Developmental Therapeutics Program at the National Cancer Institute. [b] % Inhibition = (To − Tt)/To × 100, where To represents the turbidity reading of the system in the absence of the test compound and Tt represents the turbidity reading of the system in the presence of the test compound. The turbidity readings of the inoculated control tubes (containing no test compound) were 194–200 Klett units. At least three sets of duplicate determinations were made for each compound. The duplicate values in each determination agreed within ±5 Klett units. A positive control, 6-mercaptopurine, was run with each assay. An inhibition value of 52–56% by this compound at 0.1 mg/mL, and a concurrent normal growth (no inhibitor present) of 194–200 turbidity reading in the determination were criteria of a valid assay. [c] Final concentration in the assay system. [d] Commercially available. [e] Color interfered with Klett reading in higher concentrations of the test compound. However, the tubes were clear (no turbidity) upon visual examination. [f] Anticancer compounds in development in the Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD. [g] Indicates stimulation of growth. [h] Negative control compounds: see text.

tumor systems (L1210[11] lymphoid and P388[12] lymphocytic leukemias). In addition, there are more than 160 compounds, including β-hydroxyamino acids (2, 3), *N*-chloroacetyl-β-hydroxyamino acids (2, 3), *N*-acetyl- and *N*-propionylamino acids (4), free amino acids (4), *N*-chloroacetyl amino acids (4), *N*-trifluoroacetyl amino acids (5), and certain *N*-benzoyl amino acids (6, 7) that exhibited no inhibition in this assay system. There are also ~30 compounds thus far reported (2–7) that showed considerable activity in this system. However, since these compounds, both active and inactive in the assay, have not been tested in human malignancies, no correlation between the *in vitro* data and the efficacy in human cancer can be made at this time.

Possibly the reason some of the antineoplastic compounds were "missed" in this test was due to the requirement for a metabolic conversion to an active form, and to the lack of an appropriate enzyme system in the test microorganism. For example, it has been reported that cyclophosphamide is activated by an enzymatic cleavage of the cyclic compound at the P—N-linkage (8–10) and procarbazine, upon metabolic conversion has been reported to depolymerize DNA (11). While some of the other compounds that require transformation to the active form showed inhibition in this system [such as 6-mercaptopurine (12), mitomycin (13), and cytarabine (14, 15)], the system apparently possessed the necessary mechanism to cause the transformation to the active form.

When designing prospective anticancer compounds, a simple and rapid assay procedure to follow the alteration of activity with modification in the molecular structure is necessary. Therefore, a procedure consisting of a single microbiological system was selected for studying the activity of the numerous compounds prepared in this laboratory. *Lactobacillus casei* ATCC 7469 in riboflavin assay media was selected because this system was rapid (with a 19-h incubation period) and was found to be the most sensitive among a set of three systems used in our earlier studies (2).

[11] Data obtained from Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20205.

[12] Unpublished data; screening performed through Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20205.

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