NEW ANTITUMOR AGENTS FROM BLUE-GREEN ALGAE: THE SEARCH CONTINUES

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Abstract

Over 2000 extracts of laboratory-cultured blue-green algae were screened for solid tumor and tumor selective cytotoxicity in the Corbett assay. Of the 0.8 % extracts that were solid tumor selective, the lipophilic extract of Nostoc sp. GSV224 was the most cytotoxic and one of the few extracts that were both solid tumor and tumor selective. Cryptophycin-1 was found to be the most active compound in this alga and in vivo studies indicated that it was active against a broad spectrum of solid tumors implanted in mice, including multiple-drug-resistance tumors. Several analogs of cryptophycin-1 have been prepared by total synthesis and one of them, cryptophycin-52, has been selected for human clinical trials. The search for other potentially useful antitumor agents from other extracts of laboratory-cultured and field-collected blue-green algae is continuing.

Key-words: cancer drugs, natural products, cyanophyta

In the mid 1970's my research group initiated an intensive program to screen extracts of blue-green algae (cyanobacteria) for antitumor activity (1). Over the 20 years that followed we found that 6-10% of the extracts of laboratory-cultured cyanophytes were cytotoxic against human tumor cell lines at <25 μg/mL (2, 3). An even greater percentage of extracts of field-collected cyanophytes showed comparable cytotoxicity (4). By the summer of 1988 we had accumulated a relatively large number of extracts of new blue-green algae and had isolated and identified many structurally-novel cytotoxins and cytotoxic fungicides. We needed a procedure to prioritize these leads for *in vivo* evaluation. In 1998 we began a collaboration with Drs. T. Corbett and F. Valeriote at Wayne State University (WSU) to screen pure cytotoxins and crude extracts for solid tumor and tumor selective cytotoxicity (5, 6).

Description of the Screening Procedure

Toward the goal of obtaining efficacious drugs for solid tumors, which account for most of the cancer deaths in the United States, Corbett *et al.* (7). had developed a rapid and relatively inexpensive disk-diffusion assay to find agents with greater cytotoxicity against solid tumors than leukemias. The assay was modeled after the disk diffusion assay that is commonly used in antifungal and antibacterial screens. The test sample, *e.g.* a crude extract or a pure compound, was applied to a 6 mm paper disk which was then placed on the surface of a soft agar plate containing the solid tumor and leukemia cell lines. Ideally what one wanted to visualize in this assay was the total elimination of the solid tumor cells from the plate, i.e. a zone of inhibition so large that it extended to the plate's periphery, and, concomitant with this, no zone of inhibition for the leukemia cells. For the test sample to be classified as minimally (marginally) solid tumor selective, the zone of inhibition for the solid tumor (Z_{ST}) had to be at least 7.5 mm (250 zone units) larger that the zone of inhibition for the leukemia (Z_L). A zone differential of this size (Z_{ST} - Z_L = ≥250) had been found to be of significance in predicting efficacy in mice. Initially the assay was performed by directly comparing the cytotoxicity of the test sample against the solid tumor and leukemia on the same plate. The morphologically different solid tumor and leukemia cells could be easily distinguished when the plate was examined under a microscope at the end of the assay run. Studies ultimately showed, however, that the same results could be obtained by carrying out the comparison of solid tumor and leukemia cytotoxicity on separate plates. The latter procedure is presently being used.

Each test sample is examined against five different cell types, viz. a murine leukemia (L1210 or P388), a drug-sensitive murine solid tumor (colon adenocarcinoma C38, pancreatic ductal adenocarcinoma P03 or mammary adenocarcinoma M16), a multidrug-resistant (MDR) murine solid tumor (mammary adenocarcinoma M17), a human solid tumor cell line (colon CX-1, HCT8, H116 or lung H125), and a normal cell (fibroblast L-929 or intestinal I18), in the Corbett assay. Based on zone differentials the test substance is categorized into one of five groups: (i) solid tumor selective when Z_{ST} - Z_L = ≥250, (ii) leukemia selective when Z_L - Z_{ST} = ≥250, (iii) tumor selective when Z_{ST} - Z_N or Z_L - Z_N = ≥250 (ZN is the zone of inhibition for a normal cell), (iv) equally active when Z_{ST} - Z_L = <250 and Z_{ST} or Z_L = ≥250, and (v) inactive when Z_{ST} and Z_L = <250.

The test samples that are found to be either solid tumor selective or tumor selective became candidates for *in vivo* trials in mice against appropriate, subcutaneously-implanted solid tumors of murine and human origin. Generally, the test samples are evaluated by intravenous (IV) injection. Highest priority for *in vivo* evaluation is given to test samples that are MDR solid tumor selective.

Leukemia selective or equally cytotoxic test samples are subsequently evaluated in a secondary *in vitro* assay (Valeriote Assay) to compare cytotoxicity against a tumor cell and the stem cell of hematopoietic tissue obtained from murine bone marrow (CFU-GM), a normal cell. Test samples which show a zone differential equal to or greater than 250 units in favor of the tumor cell are termed tumor selective and become candidates for *in vivo* evaluation.

Interestingly most of the antitumor compounds that we had found in the 1975-1985 period through *in vivo* screening tested positively in either the Corbett or Valeriote assays. Tubercidin, tolytoxin and the scytophycins exhibited solid tumor selective cytotoxicity in the Corbett assay, whereas aplysiatoxins, oscillatoxins and lyngbyatoxins displayed tumor selective cytotoxicity in the Valeriote assay. This evidence made us very confident that a relatively large pool of test samples could be effectively pruned and the most important leads would be found using these two assays.

In the five year period from 1989-1993, 82 pure cytotoxins, some of which were potent fungicides, and 2005 extracts were screened for solid tumor and tumor selective cytotoxicity at WSU (1). Ten of the cytotoxins (12%) and 16 of the extracts (0.8%) were identified as solid tumor selective in the Corbett assay. An additional 12 extracts (0.6%) were found to be tumor selective in the Valeriote assay.

Discovery of the Cryptophycins

Unfortunately none of the 10 solid tumor selective cytotoxins displayed significant *in vivo* activity. From one of the solid tumor selective cytotoxic extracts, however, viz. the lipophilic extract of *Nostoc* sp. GSV 224, we discovered a very important class of new antitumor agents, the cryptophycins.

Of the various cyanobacterial extracts examined through 1993, the lipophilic extract of *Nostoc* sp. GSV 224 was by far the most cytotoxic, showing MIC's of 0.24 ng/mL against KB and 6 ng/mL against LoVo. More importantly, the extract exhibited both solid tumor and tumor selective cytotoxicity in the Corbett assay. Bioassay-guided fractionation led to a fraction which was predominantly cryptophycin-1 (**C-1**) (see Chart 1), (8) a cyclic depsipeptide derived from a polyketide-type d-hydroxy acid (Unit A), an a-amino acid (Unit B), a b-amino acid (Unit C), and an a-hydroxy acid (Unit D) (Fig. 1). **C-1**, however, was a known antifungal agent that had been first isolated from *Nostoc* sp. ATCC 53789 by researchers at Merck and found to be very active against strains of *Cryptococcus* (9, 10). [*Cryptococcus neoformans* is an opportunistic fungus that infects immunodeficient patients suffering from AIDS and cancer.] Merck, however, had found **C-1** to be too toxic in animal trials for use as an antifungal agent and had lost interest in pursuing the lead further. In our hands, **C-1** showed potent cytotoxicity against human tumor cell lines at 10-20 pM, equal cytotoxicity against drug-sensitive and multidrug-resistant cell lines, (11) a vinblastine-like inhibitory activity against microtubule assembly, (11) and displayed good activity against a broad spectrum of drug-