DETECTION OF GILVOCARCIN ANTITUMOR COMPLEX BY A BIOCHEMICAL INDUCTION ASSAY (BIA)*

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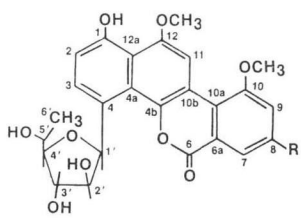
We are currently investigating the applicability of a biochemical version of the prophage induction assay (BIA)[1], a test for agents interacting with DNA, as a potential prescreen for new antitumor antibiotics. Use of the BIA led to the isolation of *Streptomyces arenae* 2064 which produced two compounds, 2064A and 2064B. While 2064A showed high activity in the BIA spot test at low fermentation broth concentrations, results of the same sample against murine P388 lymphocytic leukemia *in vivo* showed no significant prolongation of life.

We wish to report that the BIA active products from *S. arenae* 2064 are identical to the antitumor antibiotics toromycin[2] and gilvocarcins V and M[3,4] (Fig. 1), recently isolated from different *Streptomyces* species by two independent research groups, that the antibiotic components can be separated by HPLC, and that the BIA can be effectively utilized as an antitumor prescreen to detect agents that interact with DNA.

Fig. 1. Structures of gilvocarcins V and M.



Gilvocarcin	R
V	$CH=CH_2$
M	CH_3

Fermentation of *S. arenae* 2064 was initiated by adding 0.1 ml of frozen spores (2×10^9 spores/ml) to a 500-ml baffled flask containing 100 ml of seed medium consisting of: soluble starch 3.0%, sucrose 1.0%, dextrose 1.0%, soy peptone 1.5%, corn steep liquor 1.0%, K_2HPO_4 0.3%, $CaCO_3$ 0.3%, NaCl 0.1%, and 0.1% of a mineral salt solution consisting of $ZnSO_4\cdot7H_2O$ 0.28%, ferric ammonium citrate 0.27%, $CuSO_4\cdot5H_2O$ 0.005%, $MnSO_4\cdot H_2O$ 0.1%, $CoCl_2\cdot6H_2O$ 0.01%, $Na_2B_4O_7\cdot10H_2O$ 0.009%, and $Na_2MoO_4\cdot2H_2O$ 0.0002%. The seed was incubated at 28°C for 48 hours on a rotary shaker operating at 250 rpm. A 2 ml aliquot of seed was used to inoculate a 500-ml baffled flask containing 100 ml of production medium consisting of: soluble starch 3.0%,

Fig. 2. Analytical and semi-preparative HPLC chromatograms of the gilvocarcin complex.

GCV and GCM correspond to gilvocarcins V and M, respectively.

A: 30 cm×3.9 mm i.d. μBondapak C-18 analytical column (Waters); mobile phase: CH_3OH - H_2O (70: 30); flow rate: 1.5 ml/minute; detector: absorbance at 254 nm.

B: 50 cm×9.4 mm i.d. Magnum 9 semi-preparative column (Whatman); mobile phase: CH_3OH - H_2O - tetrahydrofuran (40: 45: 15); flow rate: 5.0 ml/minute; detector: absorbance at 254 nm.

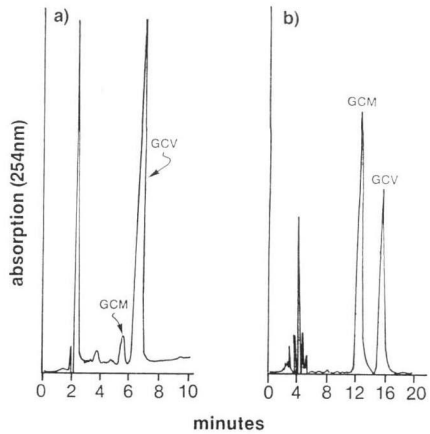


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Table 1. Physico-chemical properties.

	2064A	2064B	Gilvocarcin V	Gilvocarcin M	Toromycin
Appearance	Yellow	Yellow	Yellow	Yellow	Yellow
Melting point, °C	251~253	252~256	264~267	245~248	255~260
Molecular formula	$C_{27}H_{26}O_9$	$C_{26}H_{26}O_9$	$C_{27}H_{26}O_9$	$C_{26}H_{26}O_9$	$C_{27}H_{26}O_9$
M.S.: m/z (M^+)	494.1543	482.1569	494.1596	482.1608	494
UV λ_{max} (MeOH) nm	248, 288, 400	246, 267, 275, 390	248, 287, 398	245, 267, 275, 387	247, 277, 288, 398

Table 2. ^1H NMR comparison of 2064A and gilvocarcin V in DMSO-d_6.

Proton	2064A	Gilvocarcin V
1-OH	9.40 (1H, s)	9.66 (1H, s)
2-H	6.93 (1H, d, 8.5 Hz)	6.92 (1H, d, 8.3 Hz)
3-H	8.03 (1H, d, 8.5 Hz)	8.05 (1H, d, 8.3 Hz)
7-H	7.92 (1H, d, 1.0 Hz)	7.93 (1H, d, 1.5 Hz)
9-H	7.67 (1H, d, 1.0 Hz)	7.69 (1H, bs)
11-H	8.43 (1H, s)	8.39 (1H, s)
10-OMe	4.14 (3H, s)	4.14 (3H, s)
12-OMe	4.10 (3H, s)	4.08 (3H, s)
1′-H	6.17 (1H, d, 5.0 Hz)	6.18 (1H, d, 4.9 Hz)
2′-H	4.67 (1H, dd, 3.5 and 5.0 Hz)	4.67 (1H, m)
3′-H	3.84 (1H, m)	3.88 (1H, m)
4′-H	3.48 (1H, dd, 5.5 and 4.0 Hz)	3.53 (1H, dd, 3.9 and 5.6 Hz)
5′-H	3.84 (1H, m)	3.88 (1H, m)
6′-Me	1.25 (3H, d, 5.0 Hz)	1.26 (3H, d, 6.3 Hz)
5′-OH	5.06 (1H, d, 4.0 Hz)	5.07 (1H, d, 4.9 Hz)
3′-OH	4.80 (1H, d, 3.0 Hz)	4.83 (1H, d, 4.9 Hz)
2′-OH	4.48 (1H, d, 6.0 Hz)	4.48 (1H, d, 6.8 Hz)
13-H	6.91 (1H, dd, 11 and 17.0 Hz)	6.93 (1H, dd, 9.2 and 18.6 Hz)
14-H	5.51 (1H, d, 11.0 Hz)	5.48 (1H, d, 9.2 Hz)
14-H	6.11 (1H, d, 17.0 Hz)	6.11 (1H, d, 18.6 Hz)

peptone 0.1%, $Mg_3(PO_4)_2 \cdot 8H_2O$ 1.0%, $MgSO_4 \cdot H_2O$ 0.01%, $CaCl_2$ 0.1%. Peak antibiotic titer was obtained after 120~144 hours incubation at 28°C and 250 rpm on a rotary shaker.

The antibiotic complex was extracted from the filtered fermentation broth with ethyl acetate and from the mycelium with acetone. The combined extracts were dried and the residue chromatographed once on silica gel 60 (Merck) using 10% MeOH in $CHCl_3$ as eluant, and then on silica gel 60 H (Merck) using 5% MeOH in ethyl acetate as eluant. Concentration of the appropriate fractions gave pure 2064A.

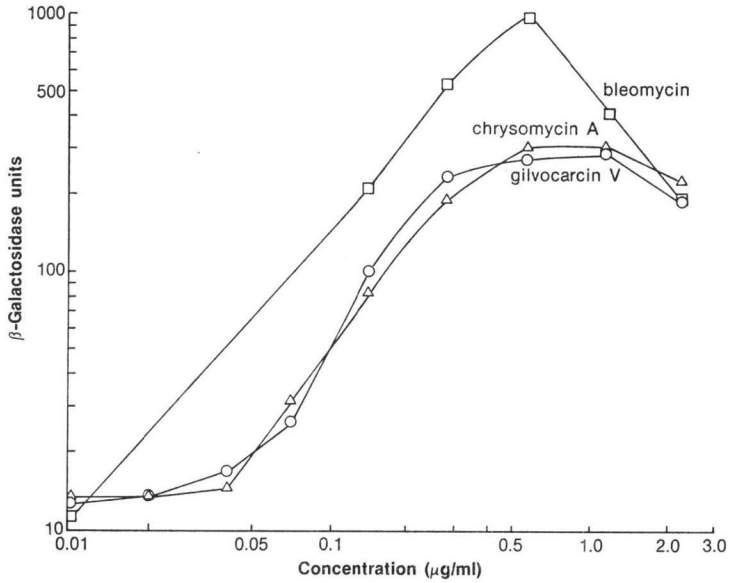
Several high performance liquid chromatographic (HPLC) systems were developed to separate 2064A and B. These were utilized for the assay of each component as well as the purification of 2064B. Analytical separations were carried out using a μBondapak C-18 column (Waters) and MeOH - H_2O (7: 3) as mobile phase (Fig. 2a). Component 2064B was isolated by pooling the appropriate fractions from the silica gel 60 H column and purifying on a C-18 Magnum 9 column (Whatman) using MeOH - H_2O - tetrahydrofuran (4: 5: 1) as mobile phase (Fig. 2b).

A comparison of the physico-chemical properties of 2064A and B, gilvocarcins V and M and

Table 3. ^{13}C NMR comparison of 2064A and gilvocarcin V in DMSO-d_6.

Carbon	2064A	Gilvocarcin V	Carbon	2064A	Gilvocarcin V
1	152.6	152.5	11	101.6	100.9
2	111.9	111.7	12	151.7	151.3
3	129.0	128.7	12a	114.9	114.5
4	126.1	125.7	13	135.3	135.0
4a	123.6	123.3	14	117.0	116.2
4b	142.2	141.9	10-OMe	56.7	56.2
6	159.5	159.3	12-OMe	56.2	55.7
6a	122.1	121.6	1′	80.9	80.8
7	119.0	118.7	2′	78.6	78.8
8	138.5	138.1	3′	78.6	78.8
9	114.4	113.8	4′	85.8	85.8
10	157.3	156.8	5′	66.4	66.5
10a	122.9	122.5	6′-Me	20.1	20.1
10b	112.8	112.5			

Fig. 3. BIA activity of gilvocarcin V and chrysomycin A determined by tube assay[1].



toromycin is presented in Table 1.* Photosensitivity of 2064A was observed under both incandescent and fluorescent light. A solution of 2064A (10 μg/ml) in tetrahydrofuran - MeOH (1 : 9) had a half-life of 50 hours under normal fluorescent room light. A comparison of the ^1H and ^{13}C NMR's of 2064A and gilvocarcin V is presented in Tables 2 and 3. An HPLC comparison of the 2064 fermentation products with authentic samples of the gilvocarcins confirmed that 2064A and B were identical to gilvocarcin V and M, respectively. While we did not have a sample of toromycin to analyze by HPLC, the

* The first of this class of compounds to be isolated was chrysomycin.[5] Chrysomycins A and B differ from 2064A (gilvocarcin V) and 2064B (gilvocarcin M), respectively, by a methyl substitution in the sugar side chain. Chrysomycin A was recently shown to exhibit antitumor activity in the P388 leukemia system.[6] Chrysomycins A and B can be separated by HPLC using the same procedures described above for the gilvocarcins.

published UV spectrum[2] with absorbances at both 277 and 288 leads us to believe that this material is really a mixture of both the vinyl and methyl components.

In addition to 2064A and B, a third component (2064C), which we believe to be the chromophore of 2064A (gilvocarcin V), was detected by HPLC. It possessed UV-visible spectral characteristics identical to 2064A, it had a retention time in our analytical HPLC system using MeOH - H_2O (76: 24) as mobile phase of 14.5 minutes compared to 4.0 and 3.5 minutes for gilvocarcins V and M, respectively, and its molecular ion was determined to be 384 ($C_{21}H_{16}O_5$) by electron impact mass spectrometry. Interestingly, this substance was also found to induce in the BIA.

The ability of the BIA to detect 2064A and B presents an example of its utility as an antitumor prescreen. Induction by gilvocarcin V in the BIA agar spot test occurred at fermentation broth concentrations in the 1~2 μg/ml range. Concentrations as low as 0.5 μg/ml of both 2064A and chrysomycin A gave induction by this technique. Even greater sensitivity was achieved by performing the BIA in liquid medium where both 2064A and chrysomycin A exhibit inflection points in enzyme activity at 0.3 and 0.6 μg/ml, respectively, compared to bleomycin at 0.6 μg/ml (Fig. 3). Such low concentrations are common in fermentation broths typically encountered in screening programs for antitumor agents. Due to the limited sensitivity of *in vivo* screens, a potentially useful drug in such a broth could be easily overlooked. Given the high sensitivity, ease of operation and low cost of the BIA, we have found this assay to be a useful supplement to our antitumor screening program.

Added in Proof

After submission of this paper gilvocarcin V was reported to be produced by another streptomycete. (BALITZ, D. M. *et al.*: Antitumor agents from *Streptomyces anandii*: gilvocarcins V, M and E. J. Antibiotics 34: 1544~1555, 1981). These authors noted that gilvocarcin V failed to induce in their lysogenic assay at concentrations up to 1.6 μg/ml, a concentration which was toxic to the host cells. We detected gilvocarcin V as an inducer in our BIA at concentrations as low as 0.3 μg/ml. When tested on agar, where a gradient of concentrations is established, toxicity of the drug did not interfere with detection.

Acknowledgements

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