KB CELL CULTURE. I. ROLE IN DISCOVERY OF ANTITUMOR AGENTS FROM HIGHER PLANTS

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ABSTRACT.—KB (Eagle) cell culture has played a powerful role in discovery of antitumor agents from higher plants. Had KB alone been used as a preliminary screen, with *in vivo* screening limited to KB-active extracts, fractions, or compounds, KB activity of crude products would have led to discovery of vinblastine, vincristine, podophyllotoxin (from which the semisynthetics VM-26 and VP—16 were derived), and all but one of the antitumor agents now under development toward or in clinical evaluation, including bouvardin, bruceantin, camptothecin, ellipticine, homoharringtonine, maytansine, taxol, thalicarpine, and tripdiolide. Indicine *N*-oxide is the only important antitumor agent which would have been discovered only by *in vivo* screening of crude plant products. A substantial number of compounds effective against lymphoid leukemia L1210 and B16 melanoma were isolated from plants, extracts of which were active against KB.

KB (Eagle) cell culture, a cell line derived from a human carcinoma of the nasopharynx (1), has been used by the National Cancer Institute (NCI) as an antitumor assay for screening plant extracts since 1960 (2). This *in vitro* assay is more sensitive to most antitumor agents than *in vivo* assays. It is also less expensive and requires less test material and time.

This paper considers the *most important* antitumor agents from higher plants to show the role of KB in their discovery or the role KB could have played in their discovery had they been unknown in 1960. These are agents which reached the decision point in development where they were considered of sufficient merit to justify preclinical evaluation in preparation for possible clinical trial. Podophyllotoxin is included because its discovery led to the important semisynthetic derivatives VP–16 and VM–26.

Screening refers here to the first assay of a crude extract or other product which suggested it may or may not contain an antitumor agent and is synonymous with "pre-screening" and "preliminary screening." An active extract or product is one which met criteria for activity specified by NCI protocols effective at the time of the assay (1).

A 1964 monograph (3) concluded that ". . . mammalian cell assays fulfill the criteria required of a useful screening tool in the search for potential antitumor agents of synthetic or natural origin. These criteria are reliability and sensitivity, so that an assay will detect most potentially useful clinical agents without an excessive number of false positives and also economy in cost." The authors favored such an assay as a pre-animal screen for a large-scale random screening program. Thus, it might be appropriate to screen first with a suitable *in vitro* assay, and carry only *in vitro*-active compounds or natural products to *in vivo* tests.

Other investigations (4–7) concluded that while KB is the *in vitro* system of choice, this assay alone is not acceptable as a screening tool because of its tendency to identify too many false positives and its failure to detect the activity of some clinically active drugs. KB emerged from these investigations as acceptable only for screening pure compounds available in amounts too small for *in vivo* assay and for monitoring fractionation of natural products which are active *in vivo* as well as *in vitro*. Thus, plant extracts are screened against KB and one or more *in vivo* systems. When an extract is active against KB and *in vivo*, the KB assay can be used to follow activity during fractionation to isolate *in vivo*-active plant constituents (8–11). Hartwell however (12), noted that "A majority of the plant derived compounds of interest including those passing the criteria . . .

for further pharmacologic and clinical study were isolated from plants originally found to be active against KB cells in culture."

By 1977, there were 38 clinically active antineoplastic drugs and 35 compounds in development (13). Others have since been selected for development, primarily analogs of known antitumor agents with better activity or less toxicity, so-called "second-generation drugs". A new plant product will not be selected for development unless it appears superior to these antitumor agents. Earlier, a new antitumor agent could advance to preclinical evaluation based on activity against one or more tumors employed for preliminary *in vivo* screening. Currently, a new agent is unlikely to advance without additional activity against one or more drug-resistant tumor-panel assays (11). The requirements a new compound must meet to be selected for preclinical evaluation are far more formidable than those required earlier. Thus, the value of KB as a tool in drug discovery is enhanced if it has played a key role in discovery of those drugs more recently selected for development.

In vivo assays most extensively employed by NCI for screening plant extracts since 1960 are sarcoma 180 (SA), adenocarcinoma 755 (CA), lymphoid leukemia L1210 (LE), Walker 256 carcinosarcoma (WA), and P388 lymphocytic leukemia (PS) (2). The latter is still used for screening plant extracts. SA and CA were discontinued in 1962 when it became evident apparent activity against these tumors was due more to animal weight loss than to a direct effect on the tumor (11). WA was discontinued in 1969 after this assay had selected several potential new drugs for clinical evaluation and its further use was not appropriate without an assessment of the clinical value of these agents (11). Furthermore, WA proved highly sensitive to tannins and phytosterols, almost ubiquitous in higher plants, which have no value as new drugs (12,14). LE is no longer employed for screening plant extracts but is predictive for clinical activity and is now used in the tumor panel. KB activity is thus further enhanced if it is predictive for LE or other tumor-panel assay activity and not predictive for SA, CA, and WA activity.

Some important plant antitumor agents were discovered before initiation of the NCI plant screening program or by NCI screening of pure compounds isolated from higher plants. The value of KB as a screening tool will be enhanced if it can be demonstrated there is a reasonable expectation these compounds would have been discovered by routine screening of crude plant extracts with KB.

Criteria for KB activity through October 1965 (15) were intended to yield about 10% active extracts (16): ED_{50} (mcg/ml) $\leqq 30$ in the first assay of an extract followed by $\leqq 20$ in a second assay with the same extract (preliminary activity) and $\leqq 20$ in a third assay with a new extract from the same plant sample (confirmation of activity). Criteria were changed in 1965 (16) to reduce number of active extracts to about 1–2% (17): $ED_{50} \leqq 15$, $\leqq 10$, and $\leqq 10$ respectively. In May 1972 criteria were changed to those initially in effect (1), and in May 1978, returned to those effected in 1965 (18). Since 1960, extracts with $ED_{50} < 1$ were regarded as preliminary active without a second assay of the original extract.

RESULTS AND DISCUSSION

Table 1 lists all but two of the *most important* antitumor agents from plants. Acronycine and *Saponaria* saponin were excluded because neither the plant extracts, screening of which led to their discovery, nor these compounds were assayed with KB. It includes two, vinblastine and vincristine, of clinical value in cancer treatment. The table provides KB-assay results for each compound. It includes NSC number and description of the plant extract, screening of which is directly responsible for discovery of that product or might reasonably be expected to lead to discovery of that product. It shows KB activity of the extract. It also shows other antitumor activity of that extract, fractions produced during isolation of active agents, or compounds listed in the first column. Data in table 1 not

TABLE 1. Screening of plant sources of important antitumor agents.[a]

Compound	KB Activity[b] ED_{50} (mcg/ml)	Extract Screened[c]	N.S.C. No.	KB Activity[d]			Other Activity[f]
				Mo/YR	ED_{50} (mcg/ml)	TSC[e]	
Acer saponin P	inactive (12)	*Acer negundo* L. ST LF FR (ETOH)	B653012	11/62	>100	02	SA [PS,WA]
Aristolochic acid	inactive (12)	*Aristolochia indica* L. RT (AQ/ETOH)	B070683	2/61 4/61 8/61	3.2 5.0 >10	11 06 02	CA [PS]
Baccharin	0.001–0.0001 (24)	*Baccharis megapotamica* Spreng. TW LF FL (AQ/ETOH)	B800157 B800157 B002	4/73 5/73 8/73	3.5 31 3.0	11 15 20C	PS [B1, LE (25)]
Bouvardin	0.00000043 (26)	*Bouvardia ternifolia* (Cav.) Schlecht. RT ST LF FL FR (AQ/ETOH)	B613763	4/65 7/65	20 26	11 06	WA [PS,9P] [B1 (34)]
		Bouvardia erecta (DC.) Standley ST LF FR (ETOH)	B819782 B819782 B002	3/75 3/75 6/75	9.6 12 8.8	11 15 20C	PS
Bruceantin	0.59 (2)	*Brucea antidysenterica* J. F. Mill. SB (AQ)	B611103 B611103 B002	4/63 5/63 6/63	21 17 17	11 15 20C	[WA] [LE,PS,B1 (12)]
Camptothecin	0.07 (27)	*Camptotheca acuminata* Decne. FR (ETOH)	B654996	not tested			LE [LE,PS,WA (12)] [B1 (28)]
		Nothapodytes foetida (Wight) Sleumer [*Mappia foetida* (Wight) Miers] PX (AQ/ETOH)	B672224 B672224 B002	9/69 10/69 6/70	<1 .27 2.3	15 15E 20C	LE,PS
Cesalin	0.02–0.025 (29)	*Caesalpinia gilliesii* (Hook.) D. Dietr. FR (AQ)	B608680	not tested			SA[WA,LL (12)]

		SD (AQ/ETOH)	B635753	7/67	<1	15	WA[PS]
				9/67	.0009	15E	
			B635753 B002	11/67	<1	20C	
Colchicine	0.0026 (5)	*Colchicum speciosum* Stev.	B631861	1/64	.23	15	[3-desmethyl colchicine: B1,
Demicolcine	0.000003 (5)	CO (ETOH)	B631861 B002	2/64	<.25	20C	LE,PS (12)]
3-desmethyl colchicine	0.024 (30)						
Ellipticine	active (12)	*Ochrosia coccinea* (Teysm. &	B656501	9/61	<1	15	LE,SA[ellipticine: B1,LE,
9-methoxy ellipticine	<1.0 (14)	Binnend.) Miq.		9/61	.63	15E	PS,WA; 9-methoxy ellipticine:
		[*Excavatia coccinea* (Teysm. &		11/61	<1	15E	LE,PS,SA (12)]
		Binnend.) Markgraf]		11/61	.36	20C	
		SB [MEOH (ALKALOID					
		FRACT)]					
		Ochrosia moorei (F. Muell.)	B656510	11/61	8.7	11	CA,LE,SA
		F. Muell.		12/61	6.9	15	
		SB (MEOH)		3/62	8.7	20C	
Emetine	active (12)	*Cephaelis acuminata* Karst.	B652174	3/61	<1	15	[PS,LE (12), B1 (31)]
		RT (AQ/ALC)		3/61	.031	15E	
				6/61	<1	20C	
Fagaronine	inactive (12)	*Fagara xanthoxyloides* Lam.	B625445	not tested			[PS] [LE (12)]
		RT (AQ/ALC)					
Holacanthone	0.029 (2)	*Castela emoryi* (Gray) Moran &	B633407	3/66	3.2	11	[WA] [B1,PS (12)]
		Felger		3/66	3.8	15	
		(*Holacantha emoryi* Gray)	B633407 B002	5/66	6.8	20C	
		ST LF FR (AQ/ETOH)					
Homoharringtonine	0.01 (32)	*Cephalotaxus harringtonia*	B602425	10/61	.021	15	[LE,PS,WA (12)]
		(Knight ex Forbes) K. Koch		11/61	3.9	20C	
		var. *drupacea*					
		(Sieb. & Zucc.) Koidzumi					
		SD (AQ)					
Indicine N-oxide	inactive (12)	*Heliotropium indicum* L.	B606134	not tested			WA[B1,PS,LE, (12) P1534 (33)]
		PL (AQ)					
Lapachol	4.4 (34)	*Stereospermum suaveolens*	B656063	not tested			WA [SA (34)]
		(Roxb.) DC.					
		RT (OIL)					

TABLE 1. Continued.

Compound	KB Activity[b] ED_{50} (mcg/ml)	Extract Screened[c]	N.S.C. No.	KB Activity[d] Mo/YR	KB Activity[d] ED_{50} (mcg/ml)	KB Activity[d] TSC[e]	Other Activity[f]
Maytansine	0.000025 (2)	*Maytenus ovata* (Wall. ex Wight and Arn.) Loes. FR (ETOH)	B631179	5/63	<1	15	PS [LE,B1 (12)]
				6/63	.063	15E	
			B631179 B002	11/63	.51	20C	
Monocrotaline	inactive (12)	*Crotalaria spectabilis* Roth FR (ETOH)	B652821	8/61	>100	02	SA
Nitidine chloride	active (12)	*Fagara macrophylla* (Oliv.) Engl. RB (AQ/ETOH)	B670942	2/65	.86	15	[LE,PS (12)]
			B670942 B002	5/65	4.3	20C	
Podophyllotoxin αPeltatin	0.022, 0.00095 (35)	*Podophyllum peltatum* L. RT RH (AQ)	B620488	1/64	<1	15	
				1/64	<1	15E	
			B620488 B002	4/64	<1	20C	
Taxol	0.000055 (36)	*Taxus brevifolia* Nutt. SB(ETOH)	B670549	4/64	<1	15	[LE,PS,WA,B1 (12)]
			B670549 B002	6/64	8.9	20C	
d-Tetrandrine dl-Tetrandrine	0.091, 0.17 (37)	*Stephania hernandiifolia* Walp. RT ALKALOID (FRAC. B-2)	B603750	11/65	5.5	11	
				11/65	.059	15	
				12/65	4.8	15E	
				12/65	10	15E	
			B603750 B002	12/65	15	20A	
				1/66	22	20N	
Thalicarpine	2.1 (14)	*Thalictrum dasycarpum* Fisch., C. A. Mey. & Ave-Lall. RT (NON PHENOLIC ALKA.)	B603790	5/66	2.1	11	[LL,PS,WA (12)]
				5/66	3.0	15	
			B603790 B002	7/66	3.7	20C	
Tripdiolide	0.001-0.0001 (38)	*Tripterygium wilfordii* Hook. f. RT (ALKALOID FRACTION)	B099943	2/70	1.9	11	[tripdiolide: LE,PS
				3/70	2.8	15	triptolide: L1,LE,PS (12)]
Triptolide	0.001-0.0001 (38)		B099943 B002	6/70	2.6	20C	

Tylocrebrine	0.0033 (5)	*Tylophora crebriflora* S. T. Blake	B613842	1/65	<1	15	[CA,LE,PS (12)]
				1/65	.032	15E	
		? (AQ/ETOH)	B613842 B002	8/65	.013	20C	
Vinblastine	0.000024–0.00038 (5)	*Catharanthus roseus* (L.) G. Don					[Clinically useful drugs]
Vincristine	0.017–0.020 (5)	(*Vinca rosea* L.)	B692122	4/60	<2.5	33	
		LF (AQ)		10/64	1.3	21	
			B692125	4/60	<17	33	
		ST (AQ)		10/64	9.8	21	
			B692128	4/60	11	11	
		RT (AQ)		10/64	5.6	21	
Voacamine	inactive (12)	*Voacanga africana* Stapf. ex G. Ell.	B606109	not tested			WA
		RB (AQ)					
		Tabernaemontana arborea J. N. Rose ex J. D. Smith	B811157	4/74	>100	02	PS[9P (20)]
		SAP					

[a]Excluding acronycine and *Saponaria* saponin.

[b]Activity of pure compound.

[c]Plant name followed by description of sample extracted and "extract" tested; data from header-card record: CO: Corm, FL: flower, FR: fruit, LF: leaf, PL: entire plant, PX: entire plant without roots, RH: rhizome, SB: stem bark, SD: seed, ST: stem, TW: twig, RB: rootbark, RT: root.

[d]Activity of crude product.

[e]Test Status Code 02,06: inactive, 11: passed stage 1 of sequential screen, 15: passed stage 2 of sequential screen (preliminary activity), 15E: additional test with extract which reached TSC/15 to establish endpoint, 20A: preliminary confirmation, 20C: activity confirmed, 20N: activity not confirmed, 21: single test assay, 33: test to be repeated (39).

[f]Data from screening data summaries unless a reference is cited. Tumors against which the crude extract was active listed first. Tumors in brackets denote activity of a fraction or pure compound. B1: B16 melanocarcinoma, CA: adenocarcinoma 755, He: HeLa human carcinoma (Cell culture), LE: lymphoid leukemia L-1210, LL: Lewis lung carcinoma, activity based on tumor weight, L1: Lewis lung carcinoma, activity based on survival, PS: lymphocytic leukemia P388 *in vivo*, 9P: P388 (cell culture), SA: Sarcoma 180, WA: Walker carcinosarcoma 256.

attributed to literature cited was compiled from NCI screening-data summaries and header-card records.

Table 1 includes 33 compounds; 15 resulted from or, if they were not previously known, would have resulted from screening plant extracts or semi-purified plant products active in KB but inactive in *in vivo* assays employed when the extract was screened: bruceantin, colchicine, demicolcine, 3-desmethyl colchicine, emetine, holacanthone, homoharringtonine, nitidine chloride, α peltatin, podophyllotoxin, taxol, thalicarpine, tripdiolide, triptolide, and tylocrebrine.

Seven compounds would have been discovered had KB alone been used for screening (i.e., the extract was active in KB and also *in vivo*): baccharin, bouvardin, camptothecin, cesalin, ellipticine, 9-methoxy ellipticine, and maytansine.

Four other compounds would almost certainly have resulted from KB screening, but the evidence is not absolute because KB screening of the extract or other product was not completed according to protocols (vincristine, vinblastine), or the product was active under earlier criteria but not under criteria in effect when screening was completed (d-tetrandrine, dl-tetrandrine). These compounds are highly active against KB.

It is reasonable to conclude that all but 7 compounds in table 1 would have been discovered had KB alone been used for screening. Fagaronine is inactive in KB but activity of the compound in the crude extract was not detected by *in vivo* assay. Crude extract B625445 was marginally active against PS (T/C= 127,135) but additional extracts failed to confirm (highest T/C=118). Alkaloids were subsequently isolated and fagaronine (B625445 K003, NSC 157995) was active against PS (T/C=270) and LE (12). Extract B656063 was not tested against KB but lapachol was marginally inactive against this assay. Lapachol has been clinically evaluted without therapeutic effect (2). Voacamine was isolated from *Voacanga africana* and subsequently submitted for antitumor screening (19). It was later isolated from *Tabernaemontana arborea* prompted by PS activity of extract B811157 (20). Fractionation was guided by activity against P388 *in vitro*. Voacamine was cytotoxic, ED_{50} 2.6 (20). Voacamine has not reached clinical evaluation nor was it in development in 1979 (2). KB inactivity of extracts containing *Acer* saponin P, aristolochic acid, and monocrotaline enhances the value of KB rather than detracts from it as these extracts were active against CA or SA, tumors which are not now highly regarded as predictors of useful clinical activity and none of these compounds are likely to become clinically useful drugs.

Indicine *N*-oxide, in clinical evaluation in 1979 (2), stands out as the only truly important antitumor agent from higher plants to which KB is insensitive and which would not have resulted from screening a crude plant extract with this assay. Isolation was prompted by WA activity and not by PS activity. Indeed, no crude extract of the source plant has been active against PS.

Table 1 includes 11 compounds active against B16 melanoma (B1), introduced in 1972 with the hope of detecting compounds clinically effective against drug-resistant slower growing solid tumors. It is now one of the tumor-panel assays employed to reach a judgement as to which agents will advance to preclinical toxicology and pharmacology (11). All but one of these compounds (indicine *N*-oxide) proved to be the active agent of a crude extract or fraction active against KB, and five (bruceantin, 3-desmethyl colchicine, emetine, holacanthone, and taxol) resulted from screening KB-active extracts which were inactive *in vivo*.

A 1976 report (12) recorded antitumor agents isolated from higher plants; 235 were tested and 202 (86%) were active against KB. Five classes were then, and remain, of keen interest because they include one or more compounds or semisynthetic derivatives which were under development, in clinical evaluation, or clinically effective in cancer treatment (2): diterpenes, lignans, quassinoids, ansa macrolides, and alkaloids. The five classes then included 134 compounds,

of which 102 were tested and 91 (89%) were active against KB. This report (12) provided, if data was then available, the T/C for each compound tested against PS, since this assay was then (and remains) the most commonly used *in vivo* assay for plant extracts. There were 63 compounds with T/C $\cong$ 150 of which 25 had a T/C $\cong$ 200; 48 were tested and 44 (92%) were active against KB. The four KB-inactive compounds are fagaronine (T/C = 270), O-methylfagaronine (T/C = 200), indicine *N*-oxide (T/C = 262), and gossypol (T/C = 150). The first and third are included in table 1. Therefore, KB is sensitive not only to a broad spectrum of antitumor agents, it is also sensitive to a great majority of those with potent PS activity. The report did not include baccharin or bouvardin as neither had then been reported as antitumor agents.

The antitumor activity of 164 species was known in 1976 to be due to tannins (12). Activity of these plants resulted primarily from screening extracts against CA, LL, SA, and WA (12). KB, as a rule, is not sensitive to tannins, agents which have no promise as useful drugs.

Phytosterols and their glycosides are of common occurrence in higher plants. The activity of 62 species had been identified by 1976 (12) as due to these constituents which also offer little promise as useful antitumor agents. By 1969 (14) the activity of 20 species had been attributed to phytosterols. The activity of 19 was against tumors other than KB, primarily WA.

Ten plant products were in advanced clinical development or Phase I or II clinical evaluation in 1979 (2). These are bouvardin, bruceantin, camptothecin, ellipticine, homoharringtonine, indicine *N*-oxide, maytansine, taxol, thalicarpine, and tripdiolide. All but indicine *N*-oxide would have been discovered had KB alone been used for screening.

KB has played a vital role in the discovery of antitumor agents from plants and could have played an even more important role if vincristine, vinblastine and podophyllotoxin had been unknown when the NCI plant screening program began in 1960. Screening with KB would have led to these three compounds, to most in table 1 which are active against LE or B1 and to all but one of those now in or under development toward clinical trial.

Most plant extracts screened by NCI were tested with at least one expensive *in vivo* assay, many against two or more. By mid-1977 more than 105,000 plant extracts had been screened (2). If the continued screening of plants is a viable avenue to discovery of new antitumor agents, and this effort continues, it is reasonable to conclude that resources devoted to screening plant extracts, had they been devoted to procurement, extraction, and KB screening of a greater diversity of plant species, would likely have led to enough new antitumor agents to more than compensate for the failure of KB to detect the activity of an extract containing indicine *N*-oxide.

I do not propose that KB should have been used as the sole assay to identify antitumor agents from plants. I do propose it would have been more effective to employ KB for screening crude extracts; initiate fractionation guided by the KB assay; employ *in vivo* assays only after KB-active agents had been concentrated, and continue fractionation only if *in vivo* activity of concentrates was established. This is the procedure followed successfully in the NCI fermentation program (21).

I question that KB should now be the *in vitro* assay of choice for antitumor screening. It is evident from table 1 that the great majority of the KB-active agents resulted from screening before 1968 and very few from subsequent screening. New discoveries are more or less inversely proportional to the number of extracts screened (22). In view of substantial evidence that the KB cell line has become contaminated with HeLa cells, including that in the American Type Culture Collection (23), it would seem appropriate to employ other *in vitro* assays now available.

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