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A RAPID AND SENSITIVE BIOASSAY INVOLVING CULTURED RAT GLIOMA CELLS TO SCREEN FOR SUBSTANCES CAPABLE OF ELEVATING INTRACELLULAR CYCLIC AMP CONCENTRATION[1]

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ABSTRACT.—Cultured rat glioma (ASK) cells are morphologically converted from a spindle to an astrocyte form when treated with dibutyryl cAMP. This morphologic transformation is discernible by light microscopy and can be visually quantitated. As described herein, dose-dependent astrocyte generation was demonstrated by treatment of confluent monolayers with forskolin [**1**], a compound known to activate adenylate cyclase, and the potency of four forskolin derivatives was found to correlate with previously established biologic potential. Neither a crude ginseng extract nor purified ginsenosides were active in the process, but supplementation of the otherwise inactive ginseng extract with **1** demonstrated 50% of the cells were morphologically converted to the astrocyte form at a concentration of approximately 0.0008%. Retinoic acid was also active in this test system; the morphologic transformation was reversed on treatment with colchicine, and intracellular cAMP concentration was elevated approximately 10-fold. Evaluation of 15 retinoids established a general correlation between the activity in this system and other systems reported in the literature. Thus, the astrocyte formation assay appears to provide several advantages that make it attractive as a screen for the detection or evaluation of substances capable of elevating intracellular cAMP concentration. In addition to technical ease, the procedure is rapid, sensitive, and relatively inexpensive.

Cyclic AMP is a crucial molecule in one of the two known second messenger intracellular signal pathways in eukaryotic cells. It occurs in a large number of phyla in the animal kingdom (2) and governs several important biochemical pathways affecting cellular growth and differentiation (3) and gene expression (4). An example of a natural product that modulates this pathway is forskolin [**1**], a labdane diterpenoid originally isolated from the roots of *Coleus forskohlii* Briq. (Labiatae) (5,6). Forskolin [**1**] is a potent, rapid, reversible activator of adenylate cyclase (7) (the enzyme responsible for the production of cAMP from ATP) and is currently being considered for use in humans as an antihypertensive agent (8). Also, the positive inotropic and platelet aggregation inhibitory activities demonstrated by this compound appear to be mediated through cAMP induction and correlate with cardiotonic and antimetastatic activities, respectively (8). In general, forskolin [**1**] has proved to be of value in characterizing the physiological role of cAMP and the molecular features of adenylate cyclase. Thus, there is clearly precedent that establishes the value of discovering agents that bear the potential of elevating intracellular cAMP concentration.

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[1]Some of the results contained herein were presented at the annual meetings of the American Society of Biological Chemists, June 7–11, 1987, Philadelphia, PA (1) and the American Society of Pharmacognosy, July 19–22, 1987, Kingston, RI.

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As with other natural product drug discovery research programs, screening of select materials (e.g., plant extracts) followed by bioassay-directed fractionation of active leads is the most logical manner of proceeding. As exemplified by responsiveness to forskolin [**1**], bioassays to detect elevation of cAMP concentration could be devised with tissue slices [e.g., rat cerebral cortical (7) or rabbit heart (9)], plasma membrane preparations [derived from a variety of tissues such as brain, heart or liver (7,10)], or blowfly salivary glands (11). With these test systems, it would be necessary to determine cAMP concentration (after treatment with the substance of interest) employing methods such as a radioimmunoassay (12) or the chromatographic procedure described by Solomon *et al.* (13). Because these processes are not trivial, it would be of value to design a bioassay that is at once fast, uncomplicated, and relatively inexpensive, concomitant with being specific in the biologic endpoint monitored, and sensitive enough to require only a small amount of test material. In the present report we describe a simple and rapid cell culture procedure in which the specific biologic endpoint studied is the elevation of intracellular cAMP concentration.

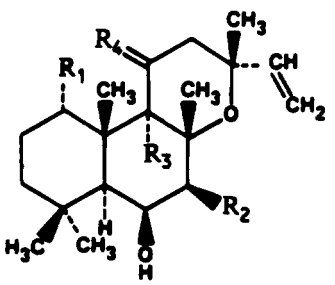
EXPERIMENTAL

CELL LINE.—Cultured rat glioma (ASK) cells were supplied through the courtesy of Dr. W. Lichter, University of Miami, Miami, Florida.

CHEMICALS.—Retinoic acid, retinyl acetate, retinol, retinal, colchicine, and sodium dodecyl sulfate were purchased from Sigma Chemical Co., St. Louis, Missouri. Retinoic acid methyl ester was prepared from retinoic acid by treatment of the latter with CH_2N_2. All other retinoids were kindly provided by Dr. W.E. Scott of Hoffmann-LaRoche, Inc., Nutley, New Jersey. Fresh retinoid solutions were prepared for each experiment, and all procedures involving these compounds were performed under subdued or yellow light.

Forskolin [**1**] and derivatives **3** and **5** (shown in Figure 1) were isolated from production material at Hoechst Pharmaceuticals Ltd., Bombay, as described previously (6). Compounds **2** and **4** were synthesized as described previously (14).

The roots of *Panax quinquefolius* L. (American ginseng) (Araliaceae) were provided by Kirkwood Associates, Chicago, based on three-year-old plants cultivated in Wisconsin. A herbarium specimen has been



Compound	R_1	R_2	R_3	R_4	ED_{50} (μM)
1	OH	OAc	OH	O	0.07
2	O—SO—	OAc	—O	O	3.12
3	OH	OH	OH	O	3.86
4	OH	OAc	OH	OH, H	6.53
5	H	OAc	H	O	>100

FIGURE 1. Structures of forskolin [**1**] and several of its derivatives with their relative activities in the astrocyte formation assay. Confluent monolayers of rat glioma (ASK) cells were incubated 1 h with various concentrations of forskolin or derivative and subsequently analyzed for astrocyte formation as described in Experimental section. Dose-response curves were generated, and doses effecting a transformation of 50% of the cells (ED_{50}) were calculated.

deposited at the Field Museum of Natural History, Chicago. An H_2O extract was prepared by extracting pulverized roots with H_2O, followed by lyophilization. Pure ginsenosides R_0, Rb_1, Rb_2, Rb_3, R_c, R_d, R_e, R_{g1}, and R_{g2} were located following the procedure of Sanada *et al.* (15). The purified ginsenosides were identified and confirmed by comparing their spectral data with those of authentic samples kindly provided by Dr. O. Tanaka.

ASTROCYTE FORMATION ASSAYS.—Cells were seeded at 10^5 per 35-mm dish (Becton Dickinson Labware, Oxnard, CA) and incubated (37°, 5% CO_2, 100% humidity) for 48 h yielding confluent monolayer cultures. The medium (Eagle's minimum essential medium supplemented with 10% fetal calf serum, GIBCO, Grand Island, New York) was either aspirated off and replaced with serum-free medium containing test substance, or an aliquot of medium was removed and an equal volume of vehicle solvent containing test substance was added (unless otherwise stated, assays were performed in serum-free medium). Varying concentrations of each substance were tested in duplicate. After a 1-h incubation period, the cells were visually examined by light microscopy for astrocyte formation.

To evaluate the nature of the astrocyte formation induced by retinoic acid, colchicine was subsequently added to these astrocyte-transformed cultures and incubated an additional hour. Percent reversal to the spindle shape was then scored.

The data presented for studies conducted with forskolin [**1**] and derivatives represent the results from one of at least two independent experiments. In each case, variability between experiments did not exceed 10%. Results concerning the structure-function relationships of retinoids (Figure 2) were derived from single experiments in which 10 concentrations of each retinoid were evaluated in triplicate. Dose-response curves were constructed from these data and ED_{50} values calculated. Due to the subjective nature which is inherent in visually estimating percent morphologic transformation of cultures, the variability of results obtained in this assay ranges approximately $\pm 10\%$. However, variability of duplicate cultures in each experiment was imperceptible.

ASSAY OF RETINOIC-ACID-INDUCED cAMP PRODUCTION.—Medium from confluent monolayer cultures of ASK cells was decanted and replaced with serum-free medium containing 50 μM retinoic acid or vehicle and incubated for 1 h. The cells were then twice rinsed with saline, harvested by treatment with 4 mM EDTA, and counted employing a hemocytometer. Four ml of 0.1 N ethanolic HCl was then added; the cells were homogenized (0–4°) and incubated at ambient temperature (5 min). Following contrifugation, the pellet was again extracted with acidic EtOH. The supernatant fractions were then combined and evaporated to dryness, and the residues were analyzed for cAMP concentration using a commercially available radioimmunoassay kit (Amersham Corp., Arlington Heights, Illinois).

RESULTS AND DISCUSSION

The ASK cells used in the current investigation have previously been employed in an assay system developed under the auspices of the National Cancer Institute for the discovery of antimitotic substances (16). The experimental endpoint in that system is also morphologic transformation of ASK cells. However, cells are first treated with dibutyryl cAMP to induce astrocyte formation and then treated with potential antimitotic agents and observed for reversion to the spindle form. Although the technique of visually estimating percent morphologic transformation is rather subjective, this method has proved to be sensitive and reproducible; the procedure has been successfully used in screening programs for the identification of antimitotic substances (17).

As described in the original report of Igarashi *et al.* (18), in addition to dibutyryl-cAMP-induced morphologic transformation, ASK cells are sensitive to compounds such as theophylline and cholera toxin. Thus, it was our reasoning that use of the first phase of this test system (i.e., astrocyte formation) could be expanded to detect the presence of substances capable of elevating intracellular cAMP concentration. Although (if successful) the procedure would not differentiate between various mechanisms of action (e.g., activation of adenylate cyclase vs. inhibition of phosphodiesterase), the anticipated simplicity of the procedure would support its utility in the discovery of compounds which could subsequently be studied in greater mechanistic detail.

As shown in Table 1, confluent monolayers are transformed to astrocyte morphology in a dose-dependent manner on treatment with forskolin [**1**], a compound well-known to activate adenylate cyclase. Moreover, as shown in Figure 1, structure-activity

TABLE 1. Astrocyte Formation of Cultured Rat Glioma (ASK) Cells Induced by Forskolin.[a]

Forskolin added (μM)	% Astrocyte formation
0	0
0.004	0
0.02	15
0.10	50
0.50	100
2.50	100

[a]Confluent monolayer cultures of ASK cells were incubated in the presence of the stated concentration of forskolin for 1 h. The plates were then visually examined for astrocyte formation.

relationships of forskolin derivatives **2–5** in the astrocyte formation assay correlate well with those observed in several in vitro assays designed to evaluate the ability of these compounds to activate adenylate cyclase (10) as well as in vivo assays measuring antihypertensive activity (19).

Cyclic-AMP-induced astrocyte formation is believed to be mediated by conformational changes in the microtubular system (18). However, it seems possible that a morphologic change similar to that induced by cAMP could be caused by a nonspecific detergent effect, rather than elevation of intracellular cAMP concentration. To test this hypothesis, confluent monolayers of ASK cells were treated with an ionic detergent, sodium dodecyl sulfate, at concentrations ranging from 5 to 200 μM. Sodium dodecyl sulfate does produce a morphologic change in the cultured cells. However, it is distinct from that induced by dibutyryl cAMP (or substances capable of elevating intracellular cAMP concentration such as **1**) both visually, as the detergent-induced transformation yields cells with poorly defined membranes, and biochemically, because this transformation is not reversed by treatment with colchicine (data not shown), a compound known to disrupt microtubule assembly (20).

An additional class of compounds reputed to affect the cAMP system is the ginsenosides (21,22). Thus, the ability of a total H_2O extract derived from *P. quinquefolius* was evaluated for potential to induce astrocyte formation with this cell system. When tested at concentrations as high as 100 μg/ml, no discernible effect on cell morphology was noted (data not shown). Similarly, purified ginsenosides (R_0, Rb_1, Rb_2, Rb_3, R_c, R_d, R_e, R_{g1}, and R_{g2}) were not effective in mediating morphological transformation when evaluated at concentrations ranging to 100 μg/ml (data not shown). Thus, although ginsenosides may alter the cAMP cascade system in some manner as reported previously, they apparently do not bind to and activate adenylate cyclase as judged by this assay system.

As an initial attempt to establish the sensitivity of the assay system, studies were then performed in which extracts of ginseng (established as inactive) were supplemented with various concentrations of **1**. As shown in Table 2, dose-dependent astrocyte formation was observed when this forskolin-supplemented extract was evaluated, and the concentration required to mediate a 50% morphologic transformation corresponded to approximately 0.0008%. The concentration of forskolin in *C. forskohlii* is reported as approximately 0.1% (6). Thus, based on this experiment, the

TABLE 2. Astrocyte Formation Mediated by Forskolin Added to an Otherwise Inactive Plant Extract.[a]

Forskolin concentration in extract (%, w/w)	% Astrocyte formation
0	0
0.00016	15
0.0008	50
0.004	85
0.02	100
0.10	100

[a]The total amount of forskolin-supplemented extract added to the culture medium corresponded to 10 mg/ml. At the highest dose tested (0.1%, w/w), the forskolin concentration corresponds to approximately 25 μM.

assay system is extremely sensitive and suitable for the detection of compounds demonstrating biological activity similar to forskolin that may occur in nature.

Additionally, we have examined the activity of vitamin A and its derivatives (retinoids) in this assay system. Recently, a number of laboratories have established that retinoic acid enhances the activity of cAMP-dependent protein kinases in mouse melanoma (B16) (23), mouse teratocarcinoma (F9) (24), and human promyelocytic (HL-60) (25) cells. Experiments by Evain *et al.* (26) demonstrate that retinoic acid increases the adenylate cyclase activity of cultured F9 cells in a time-dependent manner, and this culminates in a four-fold enhancement after 3 days. In addition, retinoic acid has been show to act synergistically with cAMP to produce differentiated phenotypes in F9 cells as evidenced by the formation of parietal endoderm (27) and induction of acetylcholinesterase activity (28). Also, priming of explants with retinoic acid in the hamster tracheal organ culture assay potentiates cAMP-mediated reversal of keratinization induced by retinoid deficiency (29). Abou-Issa and Duruibe (30), studying Sprague-Dawley rats intubated with 7,12-dimethylbenz(α)anthracene, observed that the decreased tumor incidence and growth associated with those rats fed 13-*cis*-retinoic acid or *N*-(4-hydroxyphenyl)retinamide vs. controls were accompanied by a twofold to threefold increase in tumor cell cAMP-dependent protein kinase activity. Retinoids have, thus, been shown to work in concert with the cAMP system at the cellular level (modulating enzymatic activity), the tissue level (modifying differentiation), and the level of the intact organism (affecting tumor biology).

As shown in Table 3, the morphology of the ASK cells is affected in a dose-dependent manner by retinoic acid, and this effect is diminished by the presence of serum in the medium. This diminution of activity in the presence of serum is not surprising because proteinaceous constituents such as albumin can bind retinoic acid and thereby lower its effective concentration in the medium. Thus, all subsequent assays were performed with serum-free medium to enhance the sensitivity of the assay system. Table 3 also demonstrates that the morphologic transformation caused by retinoic acid is reversible on treatment with colchicine. Thus, it appears likely that the morphologic transformation is due to elevation of cAMP concentration. However, to confirm this possibility, cells were morphologically transformed by treatment with retinoic acid and directly analyzed for their cAMP content. As summarized in Table 4, retinoic acid significantly increased cAMP content relative to controls.

TABLE 3. Astrocyte Formation of Cultured Rat Glioma (ASK) Cells Induced by Retinoic Acid in the Presence or Absence of Fetal Calf Serum (FCS) and Subsequent Reversal to the Spindle Form Upon Treatment with Colchicine.[a]

Retinoic acid added (μM)	% Astrocyte formation		% Cells existing as astrocytes after colchicine addition
	+FCS	−FCS	
6.2	0	50	0
12.5	10	70	0
25	20	80	15
50	30	95	30
100	40	100	50
150	65	toxic	

[a]Confluent monolayer cultures of ASK cells were treated with the various concentrations of retinoic acid stated yielding the respective percentage of astrocyte formation. To the cultures lacking serum (−FCS) was added colchicine (final concentration, 25 μM) and, after an additional hour, these cultures were again scored for the percentage of cells existing as astrocytes.

Figure 2 lists the relative activities of 15 retinoids in the astrocyte formation assay. With the exception of retinal, all the highly active analogues possess a free carboxyl group. These compounds include 13-*cis*-retinoic acid and 5,6-dihydroretinoic acid, which are also active in the ornithine decarboxylase assay (31). The dimethylmethoxyethylcyclopentenyl analogue of retinoic acid demonstrated considerable activity, which is consistent with its potency in the hamster tracheal organ culture assay (32). Modifications of the ring structure or polar side chain generally reduced activity. For example, etretinate, motretinide, retinoic acid methyl ester, and retinol demonstrated little activity.

As with the forskolin derivatives noted above, comparison of the structure-activity relationships observed for the retinoids in the astrocyte formation assay with those established in other test systems reveals good overall correlation. These bioassays include induction of differentiation in mouse teratocarcinoma cells (33,34) and human promyelocytic leukemia cells (35), increase of RNA in mouse epidermal cells (36), reversal of keratinization with retinoid-deficient hamster trachea (32), and inhibition of phorbol-ester-induced ornithine decarboxylase activity in mice (31). It may be noted that the concentrations of retinoids required to facilitate a response in the ASK test system

TABLE 4. Retinoic-acid-induced cAMP Production in Cultured Rat Glioma (ASK) Cells.[a]

Treatment	pmol cAMP/10^6 cells[b]
None	2.09 ± 1.33
Dimethyl sulfoxide . . .	2.41 ± 2.21
Retinoic acid	20.0 ± 2.58

[a]Confluent monolayers of ASK cells were treated with either retinoic acid (50 μM) or vehicle solvent or remained untreated. After a 1 h incubation, the cells were harvested and analyzed for intracellular cAMP concentration as described in the Experimental section.

[b]Means ± standard deviation of 4 determinations. The pmol cAMP/10^6 cells was significantly enhanced ($p<0.01$) in the presence of retinoic acid.

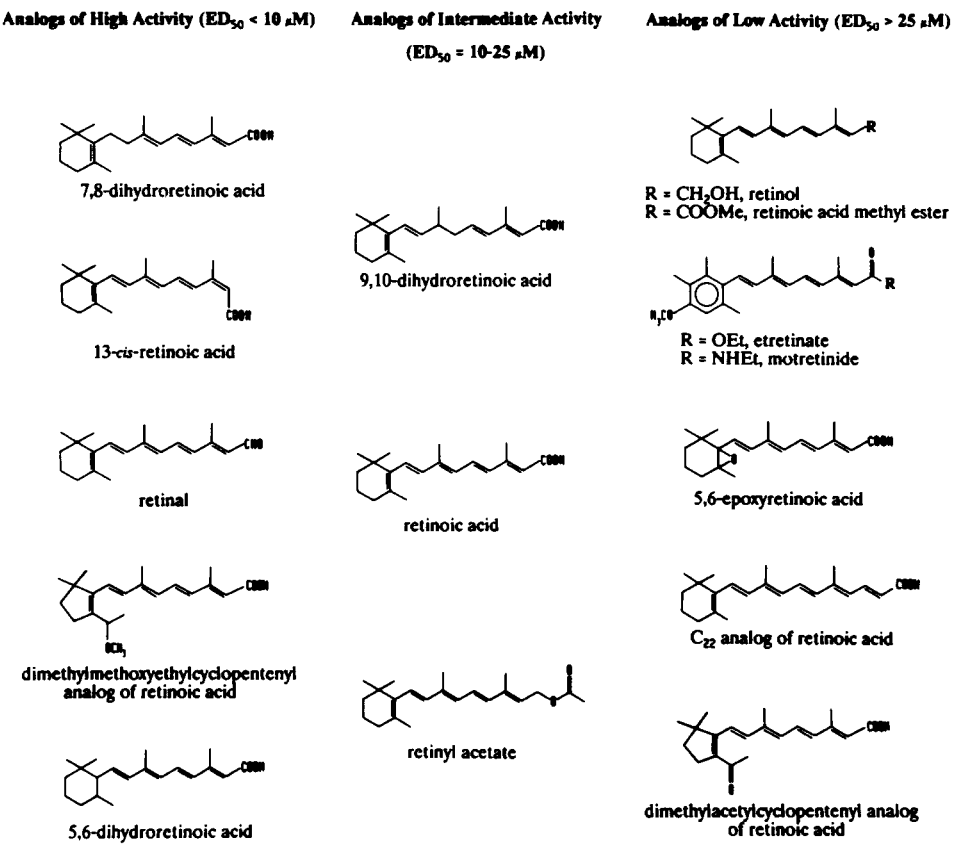


FIGURE 2. Relative activities of fifteen retinoids in the astrocyte formation assay.

are substantially higher than those required to elicit a response in the various other test systems described above. The reason for this is not known, but it may relate to the duration of the treatment period, different capacities of the test tissues to metabolize retinoids, relative affinities of retinoids for the subcellular receptor site, etc. However, the ASK system still demonstrates good sensitivity since treatment of the cells with retinoids yields ED_{50} values that are in the micromolar range.

To conclude, the assay described in this report (which we term the astrocyte formation assay) offers several important advantages which make it attractive as a screen for cAMP-inducing compounds or as a method for assessing structure-activity relationships. The assay is rapid and uncomplicated; a routine experiment involving 20 samples at five concentrations requires only a few hours of work, and the process of scoring the results demands minimal training. As a cell culture procedure, it is inexpensive relative to organ culture or in vivo methods, which may require a large number of animals to achieve statistical significance. Further, the current assay is reasonably sensitive inasmuch as dose-response relationships are observed in the micromolar range, and only a small quantity of test material (<1 mg) is required. Hence, the astrocyte formation assay holds promise as a rapid, inexpensive predictive test in programs designed for the discovery or characterization of substances capable of elevating intracellular cAMP concentration.

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