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EFFECT OF FUNGAL NATURAL PRODUCTS IN AN *AGROBACTERIUM TUMEFACIENS* POTATO DISC ASSAY

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ABSTRACT.—An *Agrobacterium tumefaciens* potato disc assay was used to screen certain natural products (25 μg/disc) from fungi for crown gall tumor/antitumor induction. Monorden (−75.0%), cladosporin (−79.0%), monocillin IV (−79.6%), duclauxin (−96.0%), diplodiol (−96.3%), and chaetoglobosin K (−99.0%) displayed concentration-dependent responses at 5, 10, 25, and 50 μg/disc. These natural products were not antimicrobial as determined by sensitivity tests using fungi and bacteria, inclusive of *A. tumefaciens*.

Fungi, and other living organisms, constitute a vast resource of natural products that may have varied agricultural, environmental, and pharmaceutical applications. Several fungal metabolites are promising antitumor agents. An example is duclauxin, produced by several *Penicillium* species (1–3), which is effective against numerous tumor cell types including Ehrlich's ascites carcinoma, lymphadenoma L-5178, HeLa, P-388, and murine leukemia L-1210 (2,4,5). The mode of action of duclauxin involves inhibition of mitochondrial respiration (prevents ATP synthesis) (2, 4–6).

Crown gall tumors on potato discs are induced by Ti plasmids in *Agrobacterium tumefaciens* and are well correlated with animal tumor cell assays as a predictor of tumor/antitumor induction, as developed by McLaughlin and his coworkers (7–9). In this study we demonstrate that duclauxin and several other natural products isolated from fungi are effective against crown-gall tumor induction. Except for duclauxin, the natural products screened are either not commercially available or have had limited prior testing as potential antitumor agents.

The results of screening certain natural products isolated from fungi for tumor/antitumor induction using the *A. tumefaciens* potato disc assay are presented in Table 1. Camptothecin, a known antitumor agent, was used as a positive control and completely inhibited tumor production. A range of responses for the natural products screened was observed. Koninginin A induced tumors by 75.0% compared to control discs while

TABLE 1. Antitumor Response for Natural Products from Fungi in an *A. tumefaciens* Potato Disc Assay.

Compound[a]	Antitumor Induction[b]
Koninginin A	+75.0
Traumatic acid	+28.4
Koninginin B	+1.0
Citrinin	−26.0
6-Pentyl-α-pyrone	−35.5
Antibiotic 1233A	−39.0
Brefeldin A	−62.0
Patulin	−66.0
Pergillin	−68.9
Monorden	−75.0
Cladosporin	−79.0
Monocillin IV	−79.6
Duclauxin	−96.0
Diplodiol	−96.3
Chaetoglobosin K	−99.0
Camptothecin	−100.0

[a]Natural products were applied as described in the Experimental. Application concentration was 25 μg/disc of each natural product.

[b]All values are relative to a control set containing no natural products as described in the Experimental. A plus (+) sign indicates tumor induction as a percent increase of observed tumors compared to a control. A minus (−) sign indicates antitumor induction as a percent decrease of observed tumors compared to a control.

chaetoglobosin K reduced tumor production (antitumor induction) by 99.0%.

Those natural products that exhibited a 75% or greater antitumor induction response in the screening assay were further tested at varying concentration levels (5, 10, 25, and 50 μg/disc). The concentration-dependent responses for monorden, cladosporin, monocillin IV, duclauxin, diplodiol, and chaetoglobosin K are shown in Table 2. Monorden, duclauxin, diplodiol, and chaetoglobosin K displayed antitumor induction at the lowest concentration (5 μg/disc) while cladosporin and monocillin IV were less effective as antitumor agents at this level.

In order to ensure that the concentration responses were due to antitumor induction by the nautral products and not to antimicrobial effects, sensitivity tests were performed as described in the Experimental. Chaetoglobosin K, diplodiol, duclauxin, and monocillin IV, at the test concentrations used did not inhibit growth of the following Gram-positive bacteria: *Bacillus subtilis, Bacillus cereus*, and *Microbacterium thermosphactum*. The following Gram-negative bacteria were also resistant to these natural products at the test concentrations used: *Escherichia coli, Enterobacterium cloacae*, and *Citrobacter freundii*. The following fungi were also resistant: *Aspergillus flavus, Curvularia lunata*, and *Chaetomium* sp. *Agrobacterium tumefaciens* was resistant to chaetoglobosin K, diplodiol, duclauxin, and monocillin IV, at each concentration tested. In summary, no zone of growth inhibition of any size was observed for any of the bacteria or fungi screened at any dose of the natural products tested. Ampicillin, chloramphenicol, and triple sulfa were employed as positive controls. The responses against *A. tumefaciens* (a Gram-negative bacterium) for the above antibiotics were susceptible, moderately susceptible, and resistant, respectively, as expected. Consequently, the observed antitumor inductions of chaetoglobosin K, diplodiol, duclauxin, and monocillin IV, were not due to biocidal or biostatic effects based on the results of the antimicrobial sensitivity tests.

TABLE 2. Dose Response by Selected Natural Products from Fungi for Antitumor Induction Using an *A. tumefaciens* Potato Disc Assay.

Compound[a]	Application Dose of Natural Product (μg/disc)			
	5	10	25	50
Monorden	−76	−94	−100	−100
Cladosporin	−14	−16	−41	−90
Monocillin IV	−8	−63	−78	−75
Duclauxin	−67	−74	−95	−94
Diplodiol	−70	−73	−97	−75
Chaetoglobosin K	−74	−97	−94	−100

[a]Natural products were applied as described in the Experimental. Results are relative to a control set containing no natural products. A minus (−) sign indicates antitumor induction as a percent decrease of observed tumors compared to a control. All percent values are given to the nearest whole number.

As determined by the *A. tumefaciens* potato disc assay, duclauxin is effective as an antitumor agent against crown gall tumors. This observation concurs with the antitumor effect reported for duclauxin against other tumor cell types and enhances the validity of the *A. tumefaciens* potato disc assay as a suitable screening technique for prospective antitumor compounds (8). Given the simplicity and low cost of this method, it should prove useful for screening crude fungal or bacterial preparations for antitumor properties as previously applied to plant extracts (9).

Monorden, cladosporin, monocillin IV, diplodiol, and chaetoglobosin K displayed antitumor induction in this assay system and each of these natural products, as observed for duclauxin, demonstrated increased antitumor induction at higher concentrations (Table 2). Furthermore, monorden, duclauxin, diplodiol, and chaetoglobosin K were effective antitumor agents at lower concentrations (5 and 10 μg/disc), while monocillin IV and cladosporin were less effective. Consequently, the potential to select antitu-

mor agents that have potent and selective cytotoxic action against tumor cells may be indicated by a concentration-dependent response in this potato disc assay.

Antimicrobial testing indicates that the effect of these natural products is antitumor and not biocidal/biostatic. By routinely incorporating antimicrobial testing in parallel with this potato disc assay, potential antitumor activity can be clearly distinguished. Based on these results, chaetoglobosin K, diplodiol, and monorden are likely prospects for further study as active agents against other tumor cell types.

EXPERIMENTAL

TEST MATERIALS.—Traumatic acid (Aldrich Chemical Company) and camptothecin (Sigma Chemical Company) were ca. 99% pure. Koninginin A and koninginin B (10,11), citrinin (12), 6-pentyl-α-pyrone (13), antibiotic 1233A (14), brefeldin A (15), patulin (12), pergillin (16), monorden (17), cladosporin (18), monocillin IV (unpublished results), duclauxin (19), diplidiol (20), and chaetoglobosin K (21) were isolated from fungal cultures as previously described.

A. TUMEFACIENS POTATO DISC ASSAY.—The *A. tumefaciens* potato disc assay for tumor/antitumor induction was performed according to the procedure of Ferrigni *et al.* (9). Potato discs were placed on 1.5% agar petri dishes. To each potato disc was applied 0.05 ml of a solution containing *A. tumefaciens* strain B_6 (48 h culture of ca. 10^9 cells/ml), DMSO/sterile H_2O and the test compound in the prescribed amounts. Control discs were prepared in the same manner except that the test compound was omitted. A minimum of three petri dishes (5 discs/dish) ($n=15$ to 25) was used for each test compound and the control. Following preparation, the petri dishes were placed in an incubator at 27° for 12 days. To determine the number of tumors, the potato discs were stained with a solution of I_2 (1 g)/KI (2 g) in 300 ml distilled H_2O for 20 min then destained in EtOH (95%) for 5 min. The tumors were counted using a dissecting microscope. For control discs: $n=15$ to 25; range of tumors per disc=0 to 75; $\mu=32$ tumors per disc; $\sigma=21$ tumors (22).

ANTIMICROBIAL SENSITIVITY TESTING.—Antimicrobial sensitivity testing of antitumor compounds was performed using the paper-disc-plate technique as previously described (17). DST agar (Oxoid) was used for the bacterial substrate while PDA (Difco) was used to support fungal growth. Petri dishes containing the appropriate medium were seeded with the selected bacterium or fungus from liquid culture. Four-mm paper discs impregnated with the test compound (50, 250, and 500 μg) dissolved in Me_2CO were then applied to the densely seeded lawns following evaporation of the solvent.

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