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Biological Effects of Cytokinin Antagonists 7-(Pentylamino) and 7-(Benzylamino)-3-Methylpyrazolo(4,3-*d*)Pyrimidines on Suspension-cultured Tobacco Cells

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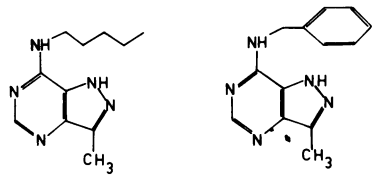
ABSTRACT

We have studied the biological effects of two structural analogs of cytokinins, 3-methyl-7-(pentylamino)pyrazolo(4,3-*d*)pyrimidine and 3-methyl-7-(benzylamino)pyrazolo(4,3-*d*)pyrimidine, on tobacco cell suspension cultures. These two cytokinin analogs are highly inhibitory to cytokinin-autonomous and cytokinin-requiring tobacco cells. The growth inhibitory effect is markedly antagonized by benzyladenine, but not by adenine. Cell suspensions of tobacco cells of a cytokinin-autonomous strain become cytokinin-dependent in the presence of 0.1 micromolar 3-methyl-7-(benzylamino)pyrazolo(4,3-*d*)pyrimidine. It is also demonstrated that growth inhibition of cell suspension cultures is accompanied by cell death and that only dividing cells are sensitive to this cytotoxic effect.

Several substituted 3-methylpyrazolo(4,3-*d*)pyrimidine analogs of cytokinins have been reported to be highly potent inhibitors of the cytokinin-dependent growth of cytokinin-requiring tobacco callus tissues. On the basis that the growth inhibition observed at a given combination of analog and cytokinin concentrations was abolished or reduced in presence of higher concentrations of cytokinins, these analogs were described as cytokinin antagonists (1). The first to be described was 3-methyl-7-(3-methylbutylamino)pyrazolo(4,3-*d*)pyrimidine (1) and the most potent in a series of 7-substituted 3-methylpyrazolo(4,3-*d*)pyrimidines described later by Skoog *et al.* (7), was 3-methyl-7-(pentylamino)pyrazolo(4,3-*d*)pyrimidine (I). Indeed, in the presence of 0.003 μM N^6-(Δ^2-isopentenyl)adenine, it was lethal at a concentration of 0.2 μM and antagonist activity was detected at the minimal concentration of 0.03 μM in the tobacco callus bioassay system (7).

Development of specific cytokinin antagonists, defined as structural analogs of cytokinins which compete with cytokinins in cytokinin-dependent biological systems (2), is of considerable potential value in studies of the mechanism of action of cytokinins. First, one experimental approach of the mechanism(s) of action of cytokinins is the identification and cellular localization of cytokinin "receptors" (6). Structural analogs of cytokinins which exhibit anticytokinin activity should be useful to assess binding activity. Second, by rendering them cytokinin-dependent if the action of the anticytokinins were reversible, the anticytokinins would extend the study of cytokinin action to biological systems which normally do not require an exogenous cytokinin. Third, there is still no certain answer to the central question: is metabolic "activation" a prerequisite for the expression of the biological activity of cytokinins such as BA or zeatin, or are they the functional molecular species? The cytokinin antagonists, by decreasing the efficiency of cytokinins in a biological system the response of which is limited by the concentration of the supplied cytokinin, should help to assess the significance of the various cytokinin metabolites which are formed if the metabolism of the supplied cytokinin(s) were modified by the cytokinin antagonists.

However, it has not been established that cytokinin antagonists act through the same metabolic pathway(s) or at the same cellular receptor site(s) as do cytokinins, and the cellular level at which they interact is also unknown. Our concern with cytokinin utilization and activity in tobacco cells grown as cell suspensions in liquid medium which are suitable, in contrast to tobacco callus tissue grown on solid medium, for study of the reciprocal interaction of cytokinins and anticytokinins at the level of their respective uptake and metabolism, led us to investigate the biological effects of two substituted 3-methylpyrazolo(4,3-*d*)pyrimidines on tobacco cell suspension cultures. We chose 3-methyl-7-(pentylamino)pyrazolo(4,3-*d*)pyrimidine (I) as a reference compound since it was the most potent anticytokinin in the series described by Skoog *et al.* (7), and 3-methyl-7-(benzylamino)pyrazolo(4,3-*d*)-pyrimidine (II) since we could expect to obtain it radiolabeled.



I II

We describe herein the biological effects of these two cytokinin structural analogs on cytokinin-requiring and cytokinin-autonomous tobacco cells grown as cell suspensions in liquid medium. Both analogs appear to be very potent inhibitors of the growth of these tobacco cell suspensions and this inhibitory effect is markedly antagonized by BA.

MATERIALS AND METHODS

CYTOKININ ANALOGS

3-methyl-7-(pentylamino)pyrazolo(4,3-*d*)pyrimidine (I) and 3-methyl-7-(benzylamino)pyrazolo(4,3-*d*)pyrimidine (II) were synthesized from 3-methyl-7-(methylthiopyrazolo(4,3-*d*)pyrimidine and the appropriate amine as described by Hecht *et al.* (1) and Skoog *et al.* (7). Purity of these cytokinin analogs was assessed by Sephadex LH_{20} chromatography and GLC. Their identities were established by MS. Furthermore, our sample of 3-methyl-7-(pentylamino)pyrazolo(4,3-*d*) pyrimidine exhibited the UV spectral characteristics described by Skoog *et al.* (7). Details will be reported elsewhere.

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TOBACCO CELL CULTURES AND MEDIA

A cytokinin-dependent strain (clone 19-3) and a cytokinin-autonomous strain (clone 13) of tobacco cells (*Nicotiana tabacum*, Wisconsin 38) originally cloned by Tandeau de Marsac and Jouanneau (8) were used. These cell lines were kindly supplied to us by Dr. Péaud-Lenoël (Centre Universitaire de Luminy, Marseille, France). Stock cell suspensions were grown according to Jouanneau and Péaud-Lenoël (5) as 100-ml cultures in 800-ml round-bottomed flasks placed on a rotary shaker (60 rpm) in a growth chamber at 26 C under white light (2,000 lux at the level of the flasks). Cells of cell line 13 were grown in medium containing 0.75 μM 2,4-D and 40 mM sucrose. Cells from the cytokinin-requiring cell line were grown in medium containing 0.2 μM 2,4-D, 0.060 μM BA, and sucrose at the growth-limiting concentration of 20 mM. These cells require a cytokinin for cell division in the sense that addition of a cytokinin to the culture medium is absolutely necessary for indefinite growth through repeated subcultures. However, suspensions of cytokinin-requiring cells initiated at high cell densities by directly diluting 15-ml aliquots of stock cell suspensions in 100 ml of cytokinin-less fresh medium would grow during one to two passages (between three and five doublings). This apparent cytokinin-independent growth of cytokinin-requiring cells of cell line 19-3 is referred in this study as "carryover" growth. Such residual growth was minimized to about one doubling of the initial cell suspension density when cells used as inoculum were washed according to the protocol described below.

BIOASSAY PROCEDURES

Cytokinin activity or growth inhibition activity was assayed with 10-ml suspension cultures grown in test tubes (20-mm diameter, 220-mm length) agitated on roller tubes (60 rpm) placed in a growth chamber at 26 C, under diffuse light.

The test compounds and BA were dissolved in ethanol at a concentration of 10 mM. These solutions were freshly diluted with distilled H_2O in order to obtain 50 μM stock solutions whose concentrations were systematically checked by measurement of UV absorption with a Cary 15 spectrophotometer. Suitable serial dilutions were prepared from these stock solutions to obtain the required quantities of test compounds or (and) BA in solution in 1 ml of distilled H_2O in the test tubes which were then autoclaved (8 min, 120 C). This procedure presumably removed the trace amount of ethanol brought with the test compounds and which anyhow was shown to be without any effect on the growth of control cell suspensions when present at the maximal concentration of 0.05% (v/v).

The test tubes were then aseptically inoculated with 9 ml of the appropriate freshly subcultured cell suspension with a laboratory-modified glass volumetric dispenser.

Cytokinin-independent Growth of Suspensions of Tobacco Cells of Cell Line 13. Fractions of 300 ml of fresh medium containing the concentrations of 2,4-D and sucrose specified above were inoculated with 33 ml of a 14-day-old stock cell suspension culture. This dilution yielded an initial wet packed cell volume of 0.35 ml/10 ml of cell suspension.

Carryover Growth of Suspensions of Cytokinin-requiring Tobacco Cells. Fractions of 300 ml of fresh cytokinin-free medium (0.2 μM 2,4-D and 40 mM sucrose) were inoculated with 45 ml of a 14-day-old cell suspension culture. This yielded an initial wet packed cell volume of 0.30 ml/10 ml of cell suspension.

Cytokinin-dependent Growth of Suspensions of Cytokinin-requiring Tobacco Cells. Cells of a 14-day-old 100-ml stock cell suspension culture were collected under sterile conditions on a 30-μm mesh nylon filter, rinsed with 100 ml of cytokinin and auxin-free fresh medium (basal medium), and resuspended in 300 ml of fresh basal medium. This cell suspension was then agitated for 1 h at 26 C. Cells were then collected on a filter as above, resuspended in 100 ml of basal medium, and 30-ml aliquots of this washed-cell suspension were used to inoculate 300-ml fractions of fresh cytokinin-free medium (0.2 μM 2,4-D and 40 mM sucrose).

After 9 days, cell suspensions were transferred into 12-ml graduated conical centrifuge tubes and centrifuged for 5 min at 700*g*. Growth was measured by the volume of the wet packed cells expressed in ml.

CELL VIABILITY ASSAY

Cell viability was determined with the erythrosine B exclusion test as described by Hugh (4). After addition of 0.1 ml of a 4 mg/ml erythrosine B solution, cells were observed under the microscope in a Nageotte cell. Three replicates of 300 cells each were observed for each determination.

RESULTS

Effects of 7-Pentylamino and 7-(Benzylamino)-3-Methylpyrazolo-(4,3-*d*) Pyrimidines on the Growth of Tobacco Cell Suspensions. As illustrated in Figure 1, I completely inhibited growth of cytokinin-autonomous tobacco cell suspensions when it was present in cytokinin-free culture medium at a concentration equal to or above 0.05 μM. At a concentration of 0.03 μM almost complete inhibition was observed but at 0.015 μM, no effect could be detected. In repeated experiments, similar results were obtained in the same narrow range of concentrations. Because of this narrow range, all experiments were performed with serial concentrations of approximatively 1.5-fold increment.

Similarly, II inhibited the growth of suspensions of cytokinin-

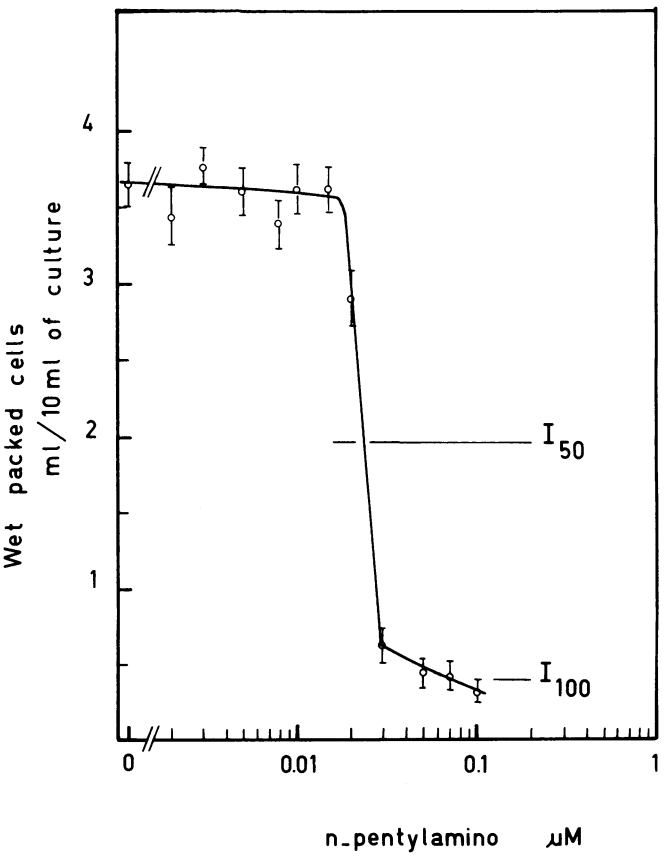

FIG. 1. Yields after 9 days of 10-ml suspension cultures of cytokinin-autonomous tobacco cells grown in the presence of serial concentrations of 3-methyl-7-(pentylamino)pyrazolo(4,3-*d*)pyrimidine (I). Each value is the mean ± SE of four replicas. I_{50} and I_{100} correspond to concentrations of I which produce 50% and near 100% of growth inhibition, respectively.

autonomous tobacco cells in about the same concentration range as I. Both analogs equally inhibited the carryover growth of cytokinin-requiring cells in cytokinin-free culture medium. The concentrations of analog which produced 50% or near 100% growth inhibition (respectively, I_{50} and I_{100}) were found to be highly reproducible from one experiment to another and therefore these parameters were chosen for the purpose of comparing the respective biological activities of I and II which are summarized in Table I.

Effect of BA on the Growth-inhibitory Effect of Cytokinin Analogs. As shown in Figure 2, inhibition of carryover growth of cytokinin-requiring tobacco cell suspension cultures produced by II was markedly antagonized by BA at low concentrations. For example, II at a concentration of 0.3 μM completely inhibited growth of cell suspensions in the absence of cytokinin, but no detectable growth inhibitory effect could be observed in the presence of 0.06 μM BA. In fact, under such conditions, almost complete growth inhibition was only obtained at a concentration of analog of 2 μM. Similarly, the growth-inhibitory effect of analogs I and II on cell suspension cultures of cytokinin-autonomous tobacco cells was antagonized by 0.1 μM BA (Table II), but apparently to a lesser extent than in the case of cell suspension cultures of cytokinin-requiring cells (Fig. 2). Adenine at the concentration of 100 μM had no effect on the growth inhibitory effect of II (Table II), indicating thereby that the cytokinin-analog antagonism is specific. BA at the concentration of 0.1 μM was a better antagonist of the benzylamino analog than of the pentylamino analog. To obtain the same level of growth inhibition in the presence of BA than in its absence, only twice as much of I was necessary whereas in the case of II, six times as much was required (Table II). Analog II, at a concentration of 0.1 μM, completely inhibited the growth of cell suspensions of cytokinin-autonomous cells in cytokinin-free medium, but was without any detectable inhibitory effect in presence of 0.1 μM BA. Hence, growth of cell suspensions of these cytokinin-autonomous cells became dependent upon the presence of a cytokinin in the culture medium when they were subcultured in the presence of II.

Table I. *Growth-inhibitory Activity of 3-Methyl-7-(Pentylamino)Pyrazolo(4,3-d)Pyrimidine (I) and 3-Methyl-7-(Benzylamino) Pyrazolo(4,3-d)Pyrimidine (II) on Suspension Cultures of Cytokinin-autonomous Tobacco Cells (Cell Line 13) and Cytokinin-requiring Tobacco Cells (Cell Line 19-3)*

Cell Line	Analog	I_{50}[a]	I_{100}
		μM	
13	I	0.025[b]	0.05[b]
	II	0.03	0.1
19-3	I	0.02	0.05
	II	0.04[c]	0.15[c]

[a] I_{50} and I_{100} correspond to concentrations of analogs which produce 50% and 100% of growth inhibition, respectively.

[b] Data obtained from the dose-response curve presented in Figure 1.

[c] Data obtained from the dose-response curve obtained in the absence of BA presented in Figure 2.

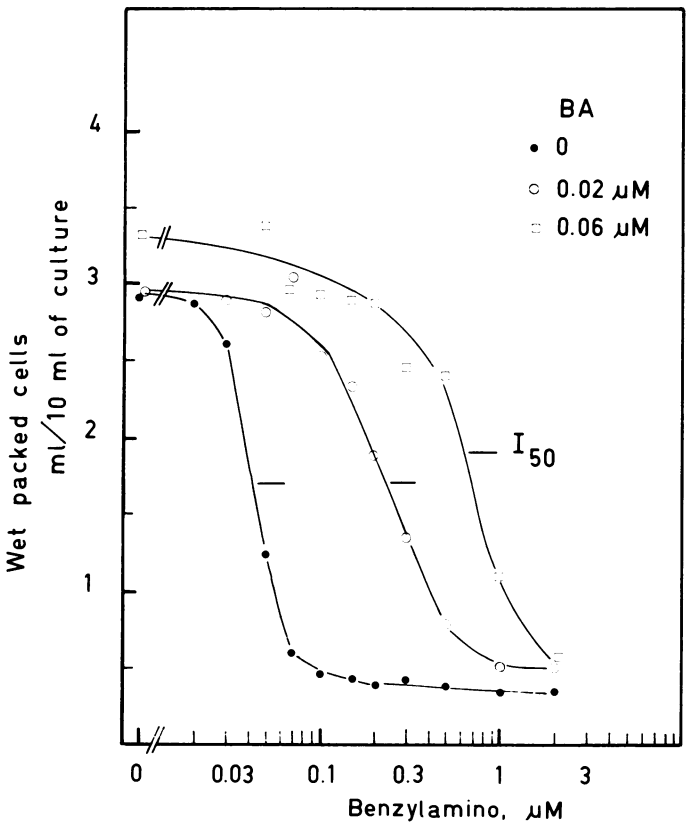

FIG. 2. Yields, after 9 days, of 10-ml suspension cultures of tobacco cells (cytokinin-requiring cell line) grown in the presence of serial concentrations of 3-methyl-7-(benzylamino)pyrazolo(4,3-*d*) pyrimidine (II) in the absence of BA (carryover growth) and in the presence of either 0.02 μM or 0.06 μM BA. Concentrations of II which produced 50% (I_{50}) and near 100% (I_{100}) of growth inhibition, respectively, were as follows. Cytokinin-free medium: 0.04 μM and 0.15 μM. In the presence of 0.02 μM BA: 0.2 μM and 1 μM. In presence of 0.06 μM BA: 0.6 μM and > 2 μM.

Effect of 3-Methyl-7-(Pentylamino)Pyrazolo(4,3-*d*)Pyrimidine on the Cytokinin-dependent Growth of Tobacco Cell Suspensions. Cytokinin-dependent growth of cytokinin-requiring tobacco cell suspensions assayed with serial concentrations of BA was almost completely inhibited by I at the concentration of 0.1 μM, even in the presence of 0.5 μM BA, while at the concentration of 0.02 μM, it exerted no inhibitory effect, except that the residual doubling of the control cell suspension cultures (cytokinin-free medium) was totally suppressed. At the intermediate concentration of 0.05 μM, I diminished the growth response of the tobacco cell suspension cultures to BA. This growth-inhibitory effect was reversible since almost full restoration of growth was attained in presence of higher concentrations of BA (Fig. 3).

Cytotoxic Effect and Growth-inhibitory Effect of the Analogs. In bioassays, inhibition of cell suspension cultures of normally green cells was accompanied by browning. Observation under the microscope revealed that most of the cells were dead. Therefore, the cytotoxicity of these analogs was investigated. As shown in Table III, cytokinin-autonomous tobacco cells died rapidly when cultured in the presence of growth-inhibitory concentrations of I. However, cell death occurred significantly later in suspension cultures containing 0.03 μM I than in suspension cultures containing 0.1 μM I. Similarly, death of most cells was observed when cytokinin-requiring tobacco cell suspensions were subcultured in the presence of growth-inhibitory concentrations of either I or II. It is evident that growth inhibition of tobacco cell suspensions by I or II resulted from the death of the cells.

Table II. *Reversal by N^6-Benzyladenine (BA) of the Growth-inhibitory Activity of 3-Methyl-7-(Pentylamino)Pyrazolo(4,3-d)Pyrimidine (I) and 3-Methyl-7-(Benzylamino)Pyrazolo(4,3-d)Pyrimidine (II) on the Growth of Tobacco Cell Suspension Cultures (Cell Line 13)*

I_{50} and I_{100} correspond to concentrations of analogs which produce 50% and 100% of growth inhibition, respectively.

Analog	I_{50}	I_{100}
	μM	
I alone	0.024	0.05
I + 0.1 μM BA	0.05	>0.1
II alone	0.03	0.1
II + 0.1 μM BA	0.2	>0.5
II + 100 μM adenine	0.03	0.1

Then, we examined the cytotoxic effect of I and II with nondividing cells in stationary phase suspension cultures of cytokinin-requiring tobacco cells. Sucrose at the concentration of 20 mM is the limiting nutrient of the growth of stock suspension cultures of these cells. At the beginning of the stationary phase of growth which occurs after 7 to 8 days of culture, cells are arrested in G_0 or G_1 phases of the cell cycle (Laloue, unpublished). Such arrested cells differentiate chloroplasts which are presumably functional, since such "green" cell suspensions remain viable for several weeks without any detectable cell division activity. Cells in stationary phase cultures appeared to be insensitive to the addition of I or II in the culture medium even at concentrations up to 5 μM (Table IV). However, when we induced cells to divide by addition of sucrose in presence of 1 μM II, 90% died between 35 and 46 h after the addition of sucrose (Table V). It seemed that dividing cells in growing cell suspension cultures were much more sensitive to the cytotoxic effect of I or II than arrested cells in stationary phase suspension cultures.

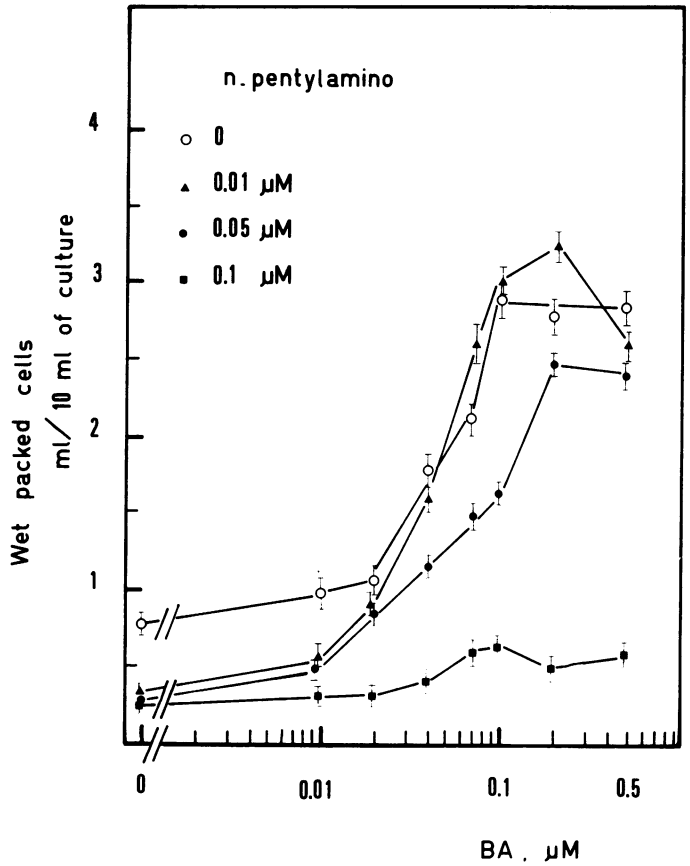

FIG. 3. Effect of 3-methyl-7-(pentylamino)pyrazolo(4,3-*d*)pyrimidine on the cytokinin-dependent growth of 10-ml suspension cultures of cytokinin-requiring tobacco cells. Wet packed cell volumes were measured after 9 days of culture. Each value is the mean ± SE of four replicas.

Table III. *Cell Mortality in Cytokinin-autonomous Tobacco Cell Suspension Cultures Grown in the Absence or the Presence of Growth-inhibitory Concentrations of 3-Methyl-7-(Pentylamino)Pyrazolo(4,3-d)Pyrimidine (I)*

	Time (days)				
	0	1	2	3	4
	No. of dead cells/100 observed cells (%)				
Control	8	14	16	12	12
Analog I 0.03 μM		12	33	43	>94
Analog I 0.1 μM		22	67	88	>97

Table IV. *Comparative Cytotoxic Activity of 3-Methyl-7-(Benzylamino)Pyrazolo(4,3-d)Pyrimidine (II) on Tobacco Cell Suspensions (Cell Line 19-3), Either Subcultured (Initial Cell Population Density: 30,000 ± 2,000 Cells/ml) or Maintained as Stationary Phase Cultures (Cell Population Density: 210,000 ± 10,000 Cells/ml) in the Presence of Analog*

	Time (days)			
	1	3	5	7
	No. of dead cells/100 observed cells (%)			
Subcultured suspension				
Without II	5	4	4	4
With II 0.2 μM	5	70	90	95
Stationary phase suspension culture				
Without II	4	4	4	4
With II 0.2 μM	4	4	4	4
With II 1 μM	4	4	4	4
With II 5 μM	4	12	23	25

Table V. *Specific Effect of Cell Division Induction in Stationary Phase Suspension Cultures of Tobacco Cells on the Expression of the Cytotoxic Activity of 3-Methyl-7-(Benzylamino)Pyrazolo(4,3-d)Pyrimidine*

At zero time, 100-ml stationary phase suspension cultures of cytokinin-requiring tobacco cells (14 days old) were diluted with 100 ml of sterile 0.2 μM Sorensen phosphate buffer (pH 7), containing 0.4 μM 2,4-D (referred as buffer), plus either sucrose and analog or sucrose or analog as indicated. Concentrations were final concentrations. Growth was measured by the wet packed cell volume method after 3 days (mean of three aliquots).

Stationary Phase Suspension Culture Diluted with:	Time (h)					Wet Packed Cell Volume
	24	35	46	72	78	
	No. of dead cells/100 observed cells (%)					*ml/10 ml of suspension*
Buffer alone	5	5	5	5	5	1.1
Buffer with 1 μM analog II	5	5	5	5	5	1.1
Buffer with 20 mM sucrose	5	5	6	8	8	2.0
Buffer with 20 mM sucrose and 0.3 μM analog II	5	5	5	22	35	1.9
Buffer with 20 mM sucrose and 1 μM analog II	5	12	80	>90	>90	1.1

DISCUSSION

Cytokinin analogs 3-methyl-7-(pentylamino)pyrazolo(4,3-*d*)-pyrimidine and 3-methyl-7-(benzylamino)pyrazolo(4,3-*d*)-pyrimidine are highly inhibitory to the growth of tobacco cell suspensions. Growth was completely inhibited in the presence of I at a concentration as low as 0.05 μM and this growth-inhibitory effect was markedly antagonized by BA. Reciprocally, I diminished the cytokinin-dependent growth of suspension cultures of cytokinin-requiring tobacco cells and this effect was reversed by BA. These results are basically in agreement with those reported by Skoog *et al.* (7) who used agar-grown tobacco callus tissue, and by Helgeson *et al.* (3) who used both agar-grown tobacco callus tissue and tobacco cells grown in liquid medium. However, our results differ in some instances at some differences in activity which may be due to cell line differences in sensitivity or/and to culture conditions. Two differences deserve to be emphasized. First, in the case of suspension cultures of cytokinin-autonomous tobacco cells, we observed a clear and potentially interesting cytokinin-antagonist interaction. Indeed, at the concentration of 0.1 μM, analog II rendered cytokinin-autonomous cells cytokinin-requiring. Such an interaction was less evident in the case of cytokinin-autonomous tobacco tissue callus as described by Skoog *et al.* (7). Second and in contrast with the results of Helgeson *et al.* (3), analogs I and II at relatively low and reversible concentrations were effective without delay as confirmed by the cytotoxicity observed.

Inhibition of the growth of tobacco cell suspensions by I or II was accompanied by cell death which occurred sooner or later, depending on the inhibitor concentration. Although such an effect may seem at first nonspecific and unrelated to the action of cytokinins on cell division, three lines of evidence suggest that these analogs may be specific anticytokinins. Cytotoxicity exhibited by I or II is markedly and specifically antagonized by BA. Also, cytotoxicity is expressed only when tobacco cells are induced to divide. Nondividing cells in stationary phase cell suspension cultures are not sensitive to I or II, and therefore, this suggests that I or II inhibits some specific biochemical event(s) during the cell cycle. Third, a high level of cell mortality is also observed in cell suspensions of cytokinin-requiring tobacco cells subcultured in cytokinin free medium after being washed (Laloue, unpublished). We are presently investigating the effect of these cytokinin analogs in relation to the progression of tobacco cells through the cell cycle.

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