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Specific recombinogenic activity of a new polyene antibiotic

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Abstract. A new antibiotic from *Streptomyces* sp., tetrapol A159, active against various fungi, a promising compound for the control of plant diseases, was studied for its genotoxic effects. It was produced at the Institute of Microbiological Preparations for Agriculture, Sofia, Bulgaria. The chemical was tested in three different test systems: a bacterial system, the Ames test for point mutations, the micronucleus test in bone marrow cells of rats for chromosomal aberrations and the fungal system of *Aspergillus nidulans* for mitotic recombination and aneuploidy. No increase in histidine revertants was observed in any of the TA100, TA98, TA1535 and TA1537 strains of *Salmonella* at concentrations ranging from 1 to 4000 mg/plate. The results were also negative in the micronucleus test of bone marrow cells at concentrations from 124 to 600 mg/kg b.w., whereas a statisically significant threefold increase of mitotic crossovers was found in *Aspergillus*, at concentrations from 0.5 to 2.5 mg/ml.

Key words. Polyene antibiotics; genotoxicity; Ames test; micronucleus test; mitotic segregation in *Aspergillus nidulans*.

Tetrapol A159 is a new polyene antibiotic deriving from *Streptomyces* sp. It was produced at the Institute of Microbiological Preparations for Agriculture, Sofia, Bulgaria, as a fungicide for the control of plant diseases. The polyenes are a group of macrolide antibiotics which alter the permeability of the membranes of sensitive cells and display a selective action against organisms whose membranes contain sterols. They are toxic against yeast, fungi and other eukaryotic cells, but they have no effect on bacteria [1].

The polyene antibiotics possess a large lactone ring containing a hydroxylated portion and a system of unsubstituted conjugated double bonds. The presence of a nonpolar (hydrophobic) and a polar (hydrophilic) region within the lactone ring renders the polyenes amphipathic. It is likely that this amphipathic feature plays an important role in the mode of action of these substances as they interact in various biological systems [2]. The cell sensitivity to polyene antibiotics is determined by the lipid composition of the plasma membrane, either in the nature of the membrane sterols or the amount present [3]. For instance, the sensitivity increases with ergosterol content in *Saccharomyces cerevisiae* [4], whereas inactivation of the ion channels in the plasma membrane induced by the polyene antibiotics is due to free radical induced–peroxidation of the polyenes [5]. The interaction of polyene antibiotics with biomembranes results in loss of intracellular protons and small molecules [6].

Among the more than 100 polyene antibiotics that have been isolated and characterized to date, some, like amphotericin B, nystatin and so on, are used in the clinical treatment of systemic fungal infections [4, 7], while several polyenes are used as fungicides in agriculture [8]. The polyenes are divided into four groups, namely tetraenes, pentaenes, hexaenes and heptaenes, depending on the number of double bonds present in the lactone ring. Tetrapol A159 is a tetraene produced for the control of fungal infections in agricultural plants.

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To our knowledge, no report has shown any genetic effects of the polyene antibiotics. In previous studies, we tested the polyenes nystatin [9] and filipin (unpublished data) in the system of *Aspergillus nidulans* for mitotic recombination, and they both proved negative.

In this work we studied the genotoxicity of tetrapol A159 in three test systems, the *Salmonella typhimurium*/mammalian microsome test for point mutations, the micronucleus test in rat bone marrow cells for chromosomal aberrations and the *A. nidulans* test for mitotic recombination and aneuploidy. The combination of these three test systems can be beneficial in identifying a wide spectrum of chemically induced genetic damage, ranging from chromosomal aberrations to point mutations, including possible effects of the chemical on the mitotic spindle or induction of recombination events.

Materials and methods

Chemicals

Tetrapol A159 was kindly provided by the Institute of Microbiological Preparations for Agriculture, Sofia, Bulgaria. It contained not less than 90% of the active ingredient, calculated with reference to dried substance. It was water-soluble and unstable under sunlight and heat. The LD_{50} was 1200 mg/kg b.w. (body weight) for male and 620 mg/kg b.w. for female rats. The chemical was dissolved in water. The compounds sodium azide, 2-nitrofluorene and 9-aminoacridine were used as positive controls in the Ames test, whereas aflatoxin B_1 was used as a positive control for the S9 mix treatment. Cyclophosphamide was used as a positive control in the micronucleus test. All these chemicals were purchased from Sigma Chemical Co., USA.

Test systems

S. typhimurium. The strains TA100, TA98, TA1535 and TA1537 of *S. typhimurium* were used. Strains TA100 and TA1535 are suitable for scoring base-pair substitutions, whereas TA98 and TA1537 are indicator strains for frameshifts. TA100 and TA98 also carry the plasmid pKM101, which makes them more sensitive to mutagenicity testing [10].

The plate incorporation technique was used as described by the Ames group [11, 12]. Bacterial cells from stock cultures were grown for 18 h in liquid medium at 37 °C. Cell suspension (0.1 ml, $2–3 \times 10^8$ cells/ml) was added in 2 ml of top agar medium containing 0.5% agar, 0.6% NaCl and traces of histidine and biotin. The chemical and the S9 mix were also added to the top agar, which was then plated on top of minimal medium. Sodium azide, 2-nitrofluorene and 9-aminoacridine were used as positive controls for the different *S. typhimurium* strains. Aflatoxin B_1 was used as a positive control for the S9 mix treatment. Three plates per dose were used in each experiment. Histidine revertant colonies were scored 48 h after inoculation and incubation at 37 °C. The results are means of three to five experiments. The chi-square test was used to check reproducibility of the experiments, and Scheffe's method was used to validate the mutagenic action of the different concentrations used (revertants/concentration), compared with the his^+ revertants of the control.

Micronucleus test. Wistar rats of both sexes weighing 150–180 g were used. Different concentrations of tetrapol A159, dissolved in water, were administered orally by a metal tube into five to seven rats per group. These concentrations corresponded to $\frac{1}{2}$ and $\frac{1}{5}$ LD_{50} (median lethal dose) in the acute and short term (subacute, 5 days) study. Concurrent negative and positive [cyclophosphamide (CPA)] control groups were used.

The procedure for slide preparation of rat bone marrow, described by Albanese and Middelton [13], was used with some small modifications in order to avoid contamination with mast cell granules, which appear to be the main artifacts in the bone marrow of this species. Slide preparations were fixed in methanol and after 24 h were stained by using Mayers Haemalaum and eosin. According to this procedure, the polychromatic erythrocytes (PEs) were stained blue-gray, and the normochromatic erythrocytes (NEs) orange-red. The micronuclei (MPEs) were stained dark blue. Slides were prepared 6, 12, 24 and 48 h after the last exposure to the compound. One thousand PEs per animal were analysed for micronuclei. The ratio of REs to NEs was determined for each animal by counting a total of 1000 erythrocytes. Student's t test was used in the statistic analysis of the results from the micronucleus test.

A. nidulans. The diploid strain of *A. nidulans* used for detecting somatic segregation has been described previously [14, 15]. It was prototrophic and heterozygous for nutritional requirements and for the white and yellow conidia color mutations. The diploid strain produced green conidia and had the genotype *suA*1*adE*20, *yA*2, *adE*20, *pabaA*1, *biA*1 (I); *AcrA*1, *wA*3, *thiA*4, *cnxE*16 (II) [14].

Minimal (MM) and complete (CM) media were used according to the routine culture techniques of Pontecorvo et al. [16]. Dilute suspensions of conidia from the diploid strain were spread on MM and incubated overnight. Blocks of inocula (1 mm^2) were then transferred to plates of CM containing the chemical under test. At least 100 colonies were tested in each chemical concentration. The diploid strain forms green conidia, and somatic segregation was detected by the appearance of sectors with either white or yellow conidia. The numbers of segregants on control plates and on treated

plates were scored after 6 days' incubation at 38 °C. For toxicity of the chemicals the colony diameter was measured 3 days after inoculation.

The segregants were thoroughly scored and included all the macroscopically visible areas of white and yellow appearing as sectors, patches or spots. Conidia from all these areas were picked up and transferred onto slants of CM for further use and tests. Haploid and diploid segregants were distinguished by their phenotype according to the genetic assay described previously [14]. Statistical evaluation of the data was carried out as described in the *Salmonella* assay.

Metabolic activation system. Wistar rats weighing 200–250 g were injected intraperitoneally with Aroclor (500 mg/kg b.w.) 5 days before sacrifice. The livers obtained were washed and homogenized, and the homogenate was centrifuged at 9800*g* for 20 min. The procedure followed was based upon Ames et al. [11] and Frantz and Malling [17]. The S9 fraction was kept at −80 °C. The S9 mix contained in a final volume of 1 ml, 0.05 ml of S9 fraction and cofactors: glucose 6-phosphate, 7.4 mM; NADP (nicotinamide adenine dinucleotide phosphate), 1.8 mM; G-6-PDH (glucose-6-phosphate dehydrogenase), 1 U; $MgCl_2 \cdot 6H_2O$, 1.8 mM; Tris, 16 mM; and sucrose, 39 mM.

Results and discussion

The results obtained in the *Salmonella* assay are shown in table 1. The concentrations of the drug used in the test system ranged from 1 to 4000 mg/plate, with and without the addition of an S9 mix.

The mutagenic response observed suggests that tetrapol A159 is not mutagenic in the *Salmonella* tester strains,

Table 1. Mutagenic activity of tetrapol A159 in the *Salmonella*/microsome assay*.

Comp.	Conc. (mg/pl)	Number of *his*$^+$ revertants/plate in *Salmonella typhimurium* strains							
		TA 100		TA 1535		TA 98		TA 1537	
		−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Tetrapol	0	118 ± 6.71	128 ± 10.29	50 ± 10.68	38 ± 7.63	35 ± 10.20	42 ± 9.52	25 ± 5.07	31 ± 5.65
A159	1	120 ± 15.54	134 ± 9.17	54 ± 6.16	39 ± 6.16	30 ± 2.05	36 ± 3.55	28 ± 4.02	37 ± 6.18
	10	121 ± 11.14	119 ± 4.96	46 ± 8.25	43 ± 2.16	26 ± 2.49	34 ± 3.09	35 ± 0.81	31 ± 4.78
	100	123 ± 13.95	116 ± 5.73	60 ± 7.31	42 ± 2.94	31 ± 1.24	38 ± 2.86	38 ± 5.50	42 ± 2.16
	1000	113 ± 3.69	136 ± 5.03	53 ± 9.93	35 ± 5.28	34 ± 6.40	40 ± 10.4	31 ± 2.66	33 ± 6.05
	2000	128 ± 3.34	133 ± 8.05	50 ± 10.49	33 ± 3.98	35 ± 10.29	40 ± 6.06	29 ± 3.97	36 ± 5.28
	3000	145 ± 18.02	159 ± 8.93	45 ± 11.90	31 ± 7.46	39 ± 7.56	38 ± 5.52	30 ± 4.05	30 ± 3.54
	4000	135 ± 7.06	146 ± 1.92	44 ± 7.55	35 ± 6.29	41 ± 9.39	40 ± 1.63	33 ± 1.50	33 ± 5.91
SA	3	778 ± 27.88	-	674 ± 20.46	-	-	-	-	-
2-NF	10	-	-	-	-	1216 ± 31.05	-	-	-
9-AA	100	-	-	-	-	-	-	703 ± 19.64	-
AF-B1	5	-	1526 ± 44.82	-	-	-	1237 ± 38.84	-	-

*Data represent mean values ± SD from three experiments.
SA, sodium azide; 2-NF, 2-nitrofluorene; 9-AA, 9-aminoacridine; AF-B1, aflatoxin B_1.

Table 2. Micronucleus test in rat bone marrow.

Dose tetrapol A159 (mg/kg)	Term of treatment	No. of animals and sex	Sampling time (h)	PEs scored	Ratio PEs/NEs	MPEs (%)
600	acute	5 M	12	5000	1.00	1.00
600	″	5 M	24	5000	0.87	1.20
600	″	6 M	48	6000	1.00	1.00
310	″	6 F	12	6000	1.25	0.83
310	″	6 F	24	6000	0.93	1.33
310	″	6 F	48	6000	0.94	1.33
240	subacute	4 M	6	4000	1.20	1.00
240	″	4 M	24	5000	1.20	1.00
124	″	5 F	6	4000	1.30	1.40
124	″	5 F	24	5000	1.11	1.20
0		4 M	24	4000	0.90	1.00
0		4 F	24	4000	0.85	1.00
CPA						
20	acute	7 M	24	7000	1.11	6.70†
	″	7 F	24	7000	0.57*	9.60†

*$P < 0.05$, †$P < 0.01$.
Subacute treatment = 5 days.
PE, polychromatic erythrocytes; NE, normochromatic erythrocytes; MPE, micronuclei; M, male; F, female; CPA, cyclophosphamide.

Table 3. Induced mitotic segregation in *Aspergillus nidulans**.

Chemical	Conc. (mg/ml)	Toxicity†	Number of mitotic segregants per 100 colonies				
			total	nondisjunctionals			mitotic crossovers
				haploid	diploid	total	
Tetrapol A159	0		25	5 ± 1.24	2 ± 0.47	7	18 ± 4.98
	0.5	4 ± 1.24	32	6 ± 1.69	1 ± 0.94	7	25 ± 3.85
	1.0	10 ± 2.94	42	9 ± 2.16	1 ± 0.47	10	32 ± 4.92
	1.5	12 ± 3.29	57‡	12 ± 2.94	1 ± 1.24	13	44 ± 11.22‡
	2.0	10 ± 2.49	52‡	8 ± 2.49	1 ± 1.41	9	43 ± 7.03‡
	2.5	17 ± 6.01	64‡	9 ± 3.29	1 ± 0.47	10	54 ± 10.78‡
Hydroquinone	0.75	2 ± 1.24	110‡	10 ± 2.05	1 ± 0.47	11	99 ± 18.63‡
Thiabendazole	0.001	12 ± 2.16	210‡	150 ± 10.42‡	34 ± 6.01‡	184‡	26 ± 5.74

*Data represent mean values ± S.D from three experiments.
†Percentage reduction of colony size, measured 3 days after inoculation.
‡Statistically significant groups, $P < 0.01$.
Hydroquinone: positive control for mitotic crossing over.
Thiabendazole: positive control for nondisjunction.

because no dose-dependent relationship was established in either TA100 and TA1535 strains, detecting base-pair substitutions or TA98 and TA1537 strains, detecting frameshifts. Furthermore, no dose-dependent increase in *his*$^+$ revertants was observed in any of the strains used with the addition of metabolic activation enzymes. Tetrapol A159 was also tested in the SOS assay in *Escherichia coli* at concentrations up to 0.1 mg/sample with and without an S9 activation system (data not shown). No increase of β-galactosidase activity was observed in the test, supporting the data obtained in the bacterial mutagenicity *Salmonella* assay.

Table 2 shows the results obtained in the micronucleus test. The acute exposure doses were 600 mg/kg b.w. in male animals and 310 mg/kg b.w. in females, corresponding to $\frac{1}{2}$ of the LD_{50}, whereas the subacute exposure doses were 240 mg/kg b.w. in males and 124 mg/kg b.w. in females, corresponding to $\frac{1}{5}$ of the LD_{50}. Dose administration was oral in all cases. Neither mode of exposure resulted in an increase of the MPEs and neither was cytotoxic (ratio PEs/NEs). No clinical signs of intoxication were seen during either study.

In table 3 the results on mitotic segregation in *A. nidulans* are presented. The concentrations of the drug tested ranged from 0.5 to 2.5 mg/ml. These concentrations induced a slight inhibitory effect – the criterion being the colony diameter – lower than 20% in all concentrations. Hydroquinone and thiabendazole were used as positive controls for mitotic crossing over and nondisjunction, respectively.

As shown in table 3, tetrapol A159 increased the frequency of mitotic segregants about 2.5 times (from 25 in the control to 64 segregants in 2.5 mg/ml). Further genetic analysis of the segregants showed that the increased segregation was mainly due to crossing over (threefold increase, from 18 in the control to 54 segregants in 2.5 mg/ml), whereas no significant increase in nondisjunctionals was detected (from 7 in the control to 13 segregants in 1.5 mg/ml).

Higher concentrations of tetrapol A159 used in the test affected the normal appearance and the green color of the growing colonies, thus making difficult the identification of color sectors.

Polyene antibiotics increase the cell membrane permeability of a number of organisms, thus promoting leaking of vital cytoplasmic constituents and resulting in cell lysis and death [1, 18]. The membrane lesions induced by polyenes are dependent on the kind of membranes being treated and the type and concentration of the polyene used: it is well known that different polyenes bind to different membrane sterols with differing affinities. For example, fungal membranes containing ergosterol are more sensitive to some polyene antibiotics than cholesterol-containing animal cell membranes (as a general rule, the antifungal activity increases with the number of double bonds in the lactone ring), whereas 3T3 cells are more susceptible to amphotericin B than are HeLa cells [19]. The interaction of the polyenes with sterol-containing plasma membranes causes impairment of membrane function, resulting in loss of intracellular monovalent and divalent cations and small molecules [6, 20]. This mechanism is not fully understood, but some molecular models proposed include formation of aqueous pores comprising a ring of 8 or 10 polyene molecules spanning the lipid bilayer and perturbations in the lipid bilayer resulting from interactions between different lipid domains [21, 22]. Other models suggest that the ability of polyene antibiotics to interfere with cell membrane constituents seems to cause no pores or holes, but results in severe changes in the physical properties of the membranes, mainly through polyene avid binding with membrane sterols. Sterols are known to stabilize membrane function and affect membrane permeability. Therefore, polyene–sterol binding induces a phase transition of the membrane from an ordered state to a melted or random state [23].

The new polyene antibiotic tetrapol A159 is toxic against various fungi. Since this promising compound was to be used for the control of plant diseases, we wanted to test its ability for genotoxic damage induction. Therefore, we studied its genotoxic effects, namely point mutations, chromosomal aberrations, mitotic recombination and nondisjunction in a battery of three test systems. Although the chemical was found to be toxic in fungi using high concentrations, we could still take advantage of the *Aspergillus* test system to study the possibility for genotoxic damage induction in relatively lower concentrations, since several chemicals show genotoxic activity in nontoxic concentrations.

In this context, tetrapol A159 was shown to cause no point mutations in *S. typhimurium* and no chromosomal aberrations in rat bone marrow cells. It was, however, found to induce a statistically significant threefold increase in mitotic crossing-over events in *A. nidulans*. This increase in relatively low concentrations of the drug is probably due to an indirect interference of tetrapol A159 with recombinational processes as a result of membrane damage and alteration of equilibrium concentrations of components, affecting topoisomerase II activity or expression.

To the best of our knowledge, this is the first report on the recombinogenic activity of polyene antibiotics. Since our present positive results for polyene induction of mitotic recombination come from a study on a lower eukaryote, it would be of interest to test the ability of polyenes to interfere with the recombinational process in higher eukaryotes.

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