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Organometallic Complexes with Biological Molecules, Part 3. *in vivo* Cytotoxicity of Diorganotin(IV)chloro and Triorganotin(IV)chloro Derivatives of Penicillin G on Chromosomes of *Aphanius Fasciatus* (Pisces, Cyprinodontiformes)

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In order to obtain a continuous source of mitotic metaphases, gill tissue of *Aphanius fasciatus* (Pisces, Cyprinodontiformes) has been successfully employed. Results gathered after exposure of fish to $R_2SnClpenG$, $R_3SnClpenGNa$, to the parents R_2SnCl_2, R_3SnCl and to penGNa (penGNa = penicillinGNa; R = methyl, butyl and phenyl) suggest that both the parent organotin(IV)chloride and organotin(IV)chloropenG derivatives are toxic while penGNa exerts no significant toxic activity. Essentially, all of the chromosome abnormalities are classifiable as irregularly staining of chromosomes, breakages, side-arm bridges or pseudochiasmata.

Keywords: Organotin(IV)chloropenG derivatives; *Aphanius fasciatus*; genotoxicity; chromosome aberration

INTRODUCTION

In an attempt to increase knowledge of semisynthetic antibiotics, several new diorganotin(IV)-chloropenicillinG and triorganotin(IV)chloropenicillinGNa derivatives have recently been prepared in our laboratory. Moreover, the genotoxicity of these compounds has been tested using *Ciona intestinalis* early-developing embryos.[1]

Since, for better evaluation of the mutagenic potential of substances, the application of several different tests is strongly recommended,[2] the present paper, as an extension of our previous research, deals with possible anomalies involving chromosome patterns.

Mutagenicity tests to detect chromosomal aberrations have been extensively developed and widely used, mainly in fish and mammals.[3–6] Owing to the orientation of research towards the aquatic invertebrates in our laboratory, we have described and quantitatively evaluated chromosomal anomalies in the testes of the mesagastropod *Truncatella subcylindrica* (Mollusca)[7] and in early developing embryos of the isopod *Anilocra physodes* (Crustacea), following exposure to organotin(IV) compounds.[8]

However, each procedure possesses its advantages but also weak points. In particular, the application of *in vivo* mutagenicity tests to invertebrate species being necessarily linked to their reproductive period, tests must be carried out only at certain times in the year. In order to overcome this difficulty, in the present report which aims to analyse, at a karyological level, the biological activity of $R_2SnClpenG$ and $R_3SnClpenGNa$ solutions (penG = penicillinG; R = methyl, butyl and phenyl), we chose to evaluate, quantitatively, chromosome abnormalities using gill tissue of *Aphanius fasciatus* (Pisces, Cyprinodontiformes) as the continuous source of metaphase spreads. Karyological analysis of early-developing embryos of *Ciona intestinalis* was not attempted due to the very minute dimensions of chromosomes in this species (about 1–2 μm in length), which prevented the unambi-

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guous recognition of possible chromosomal aberrations.[9]

EXPERIMENTAL

Material and methods

The cyprinodont *Aphanius fasciatus* inhabiting the coastal brackish waters of estuaries and lagoons,[10,11] is also readily found in some rivers of Sicily.[12]

A. fasciatus specimens employed in the present investigation were collected by net from natural populations of the Fiumicello river (Mussomeli, Caltanissetta, Sicily). Specimens were collected during many different trips to this site and were reared in the laboratory of the Istituto di Zoologia, University of Palermo, Italy.

Specimens were incubated in the presence of light, either in solutions, at different concentrations and exposure times, with the organotin(IV)chloropenG compounds and, for comparison purposes, of the corresponding parent organotin(IV)chloride and of penGNa, or in fresh water as controls (Table 1). Treated and untreated fish were injected intraperitoneally with 0.1–0.2 ml of a 0.25% colchicine solution and sacrificed 2 h later.

The gills were removed and placed in a 0.075 mol dm^{-3} KCl solution for 30 min.

The hypotonic solution was then removed and replaced with a methanol/acetic acid (3:1) solution.

After fixation for at least 30 min, the gills were immersed in 60% acetic acid and chromosome preparations were obtained using the solid tissue technique already described.[13] Chromosomes were classified according to Levan *et al.*[14].

Observations were made with a Jenamed 2 light microscope and chromosomes were photographed using Agfa Gaevaert AG 25 film.

R_2SnCl_2 or R_3SnCl (gifts from Schering AG, Bergkamen) were used after recrystallization from the appropriate solvents. PenicillinGNa (ICN Product, USA) was used without further purification, while $R_2SnClpenG$ and $R_3SnClpenGNa$ were prepared according to Maggio *et al.*[1] Test solutions of the chemicals were obtained by diluting concentrated, freshly prepared stocks. The stability of diluted solutions was checked according to procedure described elsewhere.[8]

RESULTS

Experimental conditions and results obtained after analyses of three *Aphanius fasciatus* specimens per experiment are reported in Table 1. At least two additional individuals per experiment were used as controls.

To detect possible chromosome anomalies, karyological analyses of these latter experiments were first performed. Counts of 40 spreads per individual gave diploid numbers of 48 chromosomes in all the spreads analysed, except for 4–5% which, conversely, showed chromosome values lower than the mode. In each spread, all the elements were homogeneously stained and displayed regular outlines. Only occasionally, could irregularly stained chromosomes and side-arm bridges or pseudochiasmata be observed. Chromosomes of one spread have been regularly paired and arranged, in order to decrease the length, thus forming a karyotype that included 24 mono-armed chromosome pairs [Fig. 1(a)]. The dimensions of these chromosomes ranged from 3.5 μm, for the largest to 2.3 μm for the smallest.

Moreover, the controls displayed conventionally stained metaphases which had some elements with faintly stained terminal areas interpreted as secondary constrictions, [Fig. 1(b), see arrows]. These regions gave a positive response to silver nitrate appearing as black dots, [Figs 2(a) and 2(b), see arrows]. Inter-individual variation ranging from two, [Fig. 2(a)], to four Nuclear Organizer Regions (NOR) positive chromosomes per spread, [Fig. 2(b)], was also detected. Moreover, whatever the number of observed NORs per cell, silver regions were always located terminally on the long arms of these chromosomes. However, silver stained chromosome preparations from specimens treated with $Me_2SnClpenG$ contained 93–94% of the analysed cells showing terminal NORs along with 6–7% with interstitial NORs, [Figs 3(a) and 3(b)].

Karyological analysis of specimens treated with penGNa gave results very similar to those previously described for the controls.

In comparison with the controls, specimens treated with both diorganotin(IV)chloropenG, triorganotin(IV)chloropenGNa derivatives and the parent organotin(IV)chlorides showed a substantial increase in the number of aberrant metaphases analysed by conventional staining, being higher in the triorganotin(IV) derivatives (Table 1). The most frequently observed anomaly in treated specimens consisted of irregularly stained

Table 1 Genotoxic activity: mitotic metaphase chromosomal damage in *A. fasciatus* specimens treated with R_2SnCl_2, $R_2SnClpenG$, R_3SnCl and $R_3SnClpenGNa$ (penG = penicillinG; R = methyl, butyl and phenyl)

Compound	Concentration mol dm^{-3}	Time interval (h)	No. of metaphases					
			Normal	Fragments	Irregular outlines	Pseudochiasmata	Irregular staining	Total spreads
$Me_2SnClpenG$	10^{-5}	24	4	2	30	28	40	70
		48	1	—		32	8	39
	10^{-7}	24	4	—	52	22	36	60
		48	—	3		26	6	36
Me_2SnCl_2	10^{-5}	24	2	3	18	23	22	30
		48	—	1	16	25	18	40
	10^{-7}	24	12	—	44	32	28	80
		48	8	2	40	39	38	70
$Me_3SnClpenGNa$	10^{-5}		died after a treatment of 3 h					
	10^{-7}	24	—	3	15	16	25	36
		48	—	5	22	23	32	42
Me_3SnCl	10^{-5}		died after a treatment of 3 h					
	10^{-7}	24	2	2	12	20	18	38
		48	—	5	18	24	20	40
$Bu_2SnClpenG$	10^{-5}	24	4	2	40	16	32	50
		48	4	3	30	8	36	40
	10^{-7}	24	16	5	46	4	40	80
		48	2	7	40	15	36	50
Bu_2SnCl_2	10^{-5}	24	4	—	41	16	32	50
		48	2	2	28	23	18	35
	10^{-7}	24	10	—	50	10	44	64
		48	4	2	35	8	40	50
$Bu_3SnClpenGNa$	10^{-5}		died after a treatment of 3 h					
	10^{-7}	24	2	3	12	15	20	30
		48	—	5	14	18	24	28
Bu_3SnCl	10^{-5}		died after a treatment of 3 h					
	10^{-7}	24	2	30	18	36	28	46
		48	died after a treatment of 40 h					
$Ph_2SnClpenG$	10^{-5}	24	8	4	50	8	30	60
		48	1	6	9	8	8	10
	10^{-7}	24	2	—	42	29	22	50
		48	—	—	48	27	25	58
Ph_2SnCl_2	10^{-5}	24	—	3	15	6	15	23
		48	—	—	19	10	18	25
	10^{-7}	24	2	—	10	4	10	20
		48	—	4	12	12	15	22
$Ph_3SnClpenGNa$	10^{-5}		died after a treatment of 3 h					
	10^{-7}	24	—	2	8	10	8	12
		48	—	3	10	14	12	18
Ph_3SnCl	10^{-5}		died after a treatment of 3 h					
	10^{-7}	24	3	12	15	32	36	50
		48	—	13	18	25	40	45

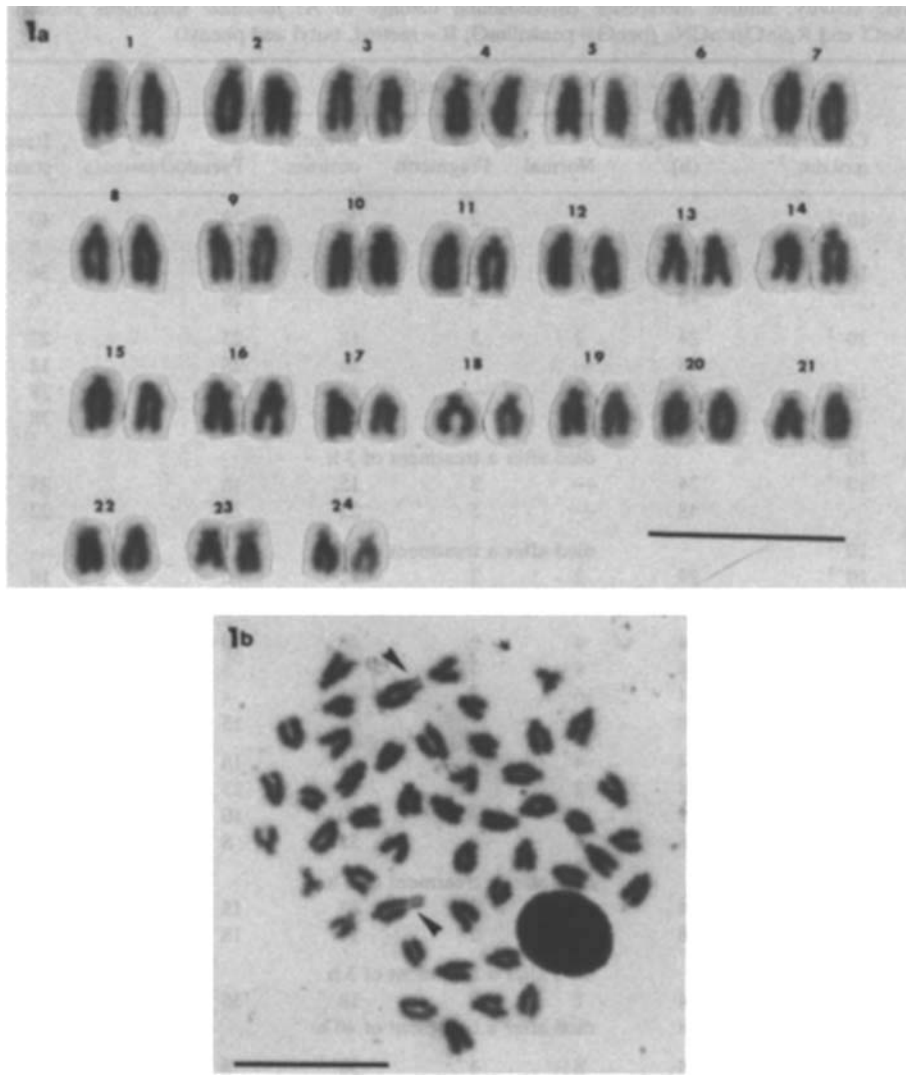

Figure 1 Giemsa stained metaphase chromosomes of control *A. fasciatus*: (a) representative karyotype; and (b) metaphase spread (arrows indicate chromosomes with terminal faintly stained regions). Bar = 10 μm.

chromosomes due to the presence of more or less large, faintly stained areas [Fig. 4(a)] along the chromosomal body. Additional anomalies included:

1. Chromosomes (one or more per spread) with small, deeply stained granular areas along the entire chromosomal arm [Fig. 4(b)].
2. Breakages [Fig. 4(c)];
3. Side-arm bridges or pseudochiasmata involving numerous elements per spread [Fig. 4(e)]
4. Spreads with all elements closely associated in groups [Fig. 5].

Finally, it must be pointed out that specimens incubated with 10^{-5} mol dm^{-3} triorganotin(IV) derivatives died (Table 1).

As in the controls, and also in treated specimens, 48 chromosomes were consistently found in all the spreads analysed, except for a small number of metaphases (4–5%) which possessed chromosome values ranging from $2n = 43$ to $2n = 47$.

DISCUSSION

Chromosome analyses made on specimens used as controls revealed a spontaneous background of abnormal metaphase figures of approximately 6–

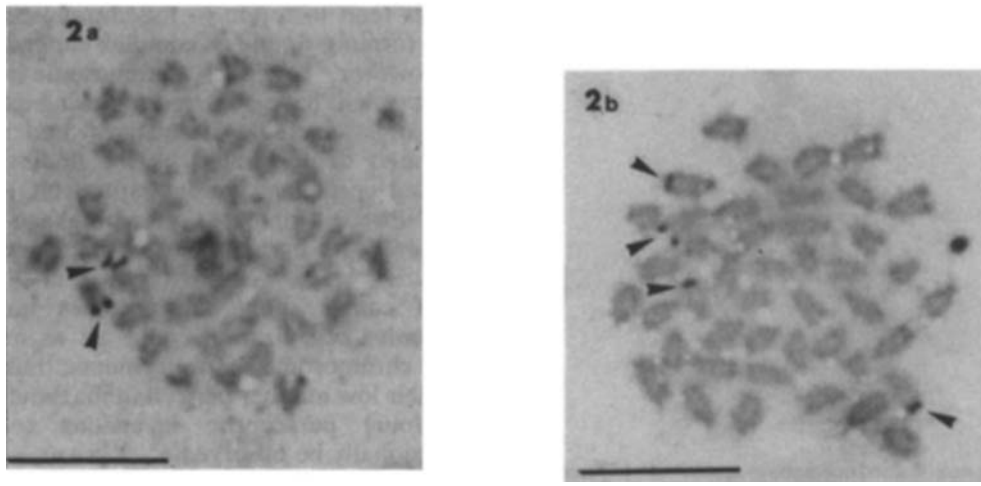

Figure 2 Silver stained metaphase spreads of control *A. fasciatus*: (a) with two NORs (arrows); and (b) with four NORs (arrows). Bar = 10 μm.

7%. The majority of the latter consisted of aneuploid cells, 4–5%, while a small percentage displayed chromosome aberrations such as irregularly stained chromosomes, breakages and pseudochiasmata.

Since the percentage of aneuploid cells were nearly identical in both treated and untreated specimens, and chromosome values observed in these cells were always lower than the mode, aneuploidy must be considered as a technical artifact rather than an anomaly due to the organotin(IV) compounds under investigation. Moreover, according to Dean and Denford[15] only hyperdiploidy, documented by the presence of

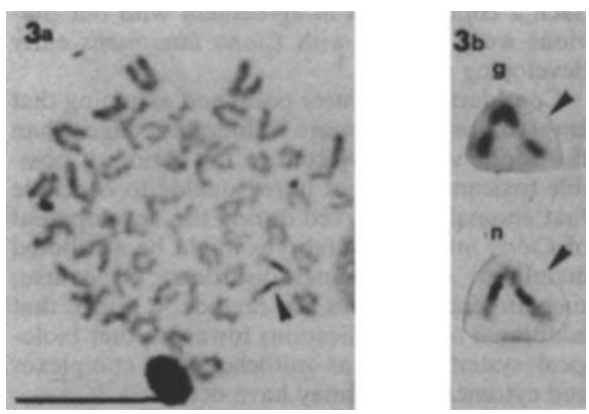

Figure 3 Silver stained metaphase spreads of *A. fasciatus* treated with 10^{-7} mol dm^{-3} $Me_2SnClpenG$; (a) metaphase spread; and (b) NOR chromosome (arrows indicates interstitial silver stained region; g = giemsa stained and n = silver stained). Bar = 10 μm.

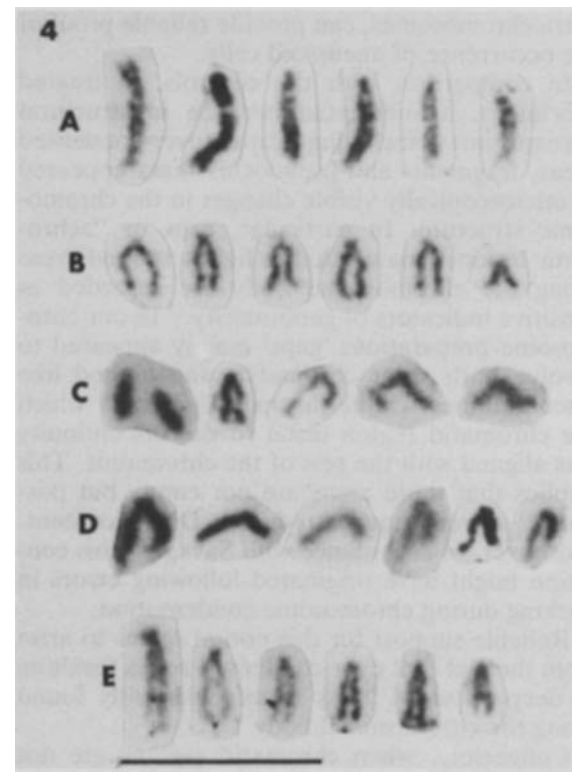

Figure 4 Examples of chromosome aberrations obtained from different giemsa stained spreads of treated *A. fasciatus* specimens: (A) = chromosomes with irregular staining; (B) = chromosomes with black granular regions; (C) = breakages; (D) = chromosomes with arms different in length; and (E) = chromosomes with pseudochiasmata. Bar = 10 μm.

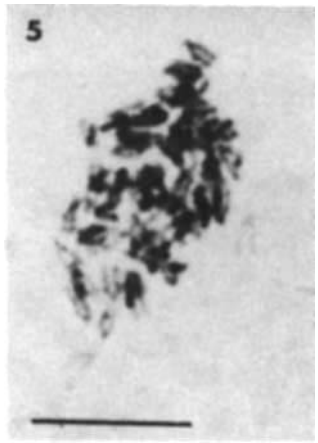

Figure 5 Giemsa stained metaphase spread of *A. fasciatus* treated with 10^{-7} mol dm^{-3} $Ph_2SnClpenG$ with chromosomes associated in groups. Bar = 10 μm.

extra chromosomes, can provide reliable proof of the occurrence of aneuploid cells.

In comparison with the controls, in treated specimens, a substantial increase in structural chromosomes including gaps, over-condensed areas, fragments and pseudochiasmata appeared as microscopically visible changes in the chromosome structure. In particular, gaps or "achromatic lesions" manifested as lightly stained areas along the chromosomal body are regarded as sensitive indicators of genotoxicity.[15] In our chromosome preparations 'gaps' mainly appeared to involve both sister chromatids and looked like discontinuities within chromatid arms, in which the chromatid region distal to the discontinuity was aligned with the rest of the chromatids. This implies that these areas are not empty but possesses a very much reduced DNA content. Moreover, in accordance with Savage,[16] this condition might have originated following errors in packing during chromosome condensation.

Reliable support for this notion seems to arise from the fact that over-condensed zones resulting in deeply stained areas were consistently found along the chromosomal body.

Conversely, when chromatid regions are not aligned, or where there is no evidence of linking strands of material across the discontinuity, gaps are regarded as complete breaks.

Unfortunately, events of this kind could only partially be documented using conventional staining. It is known, in fact, that chromosome fragments tend to rejoin to the chromosomal body, thus forming simple or complex configurations.[15]

However, that such chromosome rearrangements might have occurred following exposure to the organometallic derivatives under investigation is demonstrated by the finding, only in treated specimens, of interstitial NORs, which undoubtedly originated after breakage from terminal NORs by a process of paracentric inversion.

Of course, only chromosomes involved in nucleolus organization could act as markers of such chromosome rearrangements; thus, because of their low number per cell (a maximum number of four) paracentric inversions could only occasionally be observed.

Moreover, since there is no reason to consider NOR elements structurally different from the other chromosomes of the kayotype, the number of breakages that actually occur in treated specimens have certainly been underestimated.

Two other interesting observations can be drawn from the results of this investigation.

The first is that both the parent organotin(IV)chloride and the organotin(IV)chloropenG derivatives are toxic while penGNa exerts no significant toxic activities. This might indicate that the toxicity of these compounds is due to the action of the heavy metal contained in the organometallic derivatives. On the other hand, a variety of chromosomal aberrations have been described in fish and mammal species following exposure to heavy metals.[17]

The second is that triorganotin(IV) derivatives are more toxic than diorganotin(IV) derivatives. Such a conclusion is in agreement with our previous work dealing with *Ciona intestinalis* early developing embryos.[1]

Considering this latter point and assuming that embryos represent more vulnerable stages than the adults when subjected to the action of possible toxicants, it is not unreasonable to conclude that anomalies observed during the development of *Ciona intestinalis* embryos might be attributed mainly to chromosomal disorders. Of course, such an assertion does not exclude, a priori, that additional toxic implications towards other biological systems such as mitochondrial complexes and cytomembranes may have occurred.[18]

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