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Organometallic Complexes with Biological Molecules: V. *In vivo* Cytotoxicity of Diorganotin(IV)–Amoxicillin Derivatives in Mitotic Chromosomes of *Rutilus rubilio* (Pisces, Cyprinidae)

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In order to test *in vivo* cytotoxicity of diorganotin(IV)–amoxicillin (amox) derivatives, mitotic chromosomes of *Rutilus rubilio* (Pisces, Cyprinidae) have been analyzed using two different chromosome-staining techniques.

Results gathered after exposure of fish to the free amox · $3H_2O$, $R_2SnClamox \cdot 2H_2O$, and $R_2Snamox_2 \cdot 2H_2O$ (R = methyl, butyl and phenyl; $amox^-$ = 6-[D(−)-β-amino-*p*-hydroxyphenylacetamido]penicillinate) suggest that methyl derivatives seem to exert a lower cytotoxicity than butyl and phenyl ones and that $R_2Snamox_2 \cdot 2H_2O$ derivatives are more toxic than $R_2SnClamox \cdot 2H_2O$ at both 10^{-5} and 10^{-7} mol dm^{-3} concentrations.

The following structural lesions have been identified by comparative analysis of mitotic chromosomes from untreated specimens (controls) and specimens treated with diorganotin(IV)-amoxicillin derivatives: (1) differentially stained chromosome areas; (2) granular deeply stained zones along the chromosomal body; (3) arm breakages; and (4) side-arm bridges (pseudochiasmata).

Keywords: diorganotin(IV)chloroamoxicillin; diorganotin(IV)amoxicillin$_2$ derivatives; *Rutilus rubilio*; genotoxicity; chromosome aberration

INTRODUCTION

The policy of our research is to increase the knowledge on organotin(IV) derivatives of semisynthetic antibiotics.[1–3] Therefore, new substances are prepared in our labortory and their possible cytotoxicity is tested by *in vivo* experiments.

In an attempt of this kind, adverse effects have been analyzed in early-developing embryos of *Ciona intestinalis* (Ascidiacea)[1,4,5] in response to exposure to organotin(IV) compounds. Similarly, chromosome aberrations have been evaluated quantitatively in spermatocytes of the mesogastropod *Truncatella subcilindrica* (Mollusca)[6] and in early-developing embryos of the isopod *Anilocra physodes* (Crustacea)[7] following exposure to the same toxicants.

Mutagenicity tests have also been successfully carried out in cyprinodont *Aphanius fasciatus*[2] using gill tissues of this fish as a continuous source of metaphase spreads.

Novel diorgano- and triorgano-tin(IV)–amoxicillin derivatives ($amoxicillin^- = amox^- =$ 6-[D(−)-β-amino-*p*-hydroxyphenylacetamido]penicillinate) have been prepared recently in our laboratory; the latter decomposed in polar solvent.[3]

The aim of the present research is to test the possible cytotoxicity of diorganotin(IV)–amoxicillin derivatives by analyzing the mitotic chromosomes of the cypriniform *Rutilus rubilio*. The fish species has been chosen for our study for three reasons: (1) *R. rubilio* specimens are small in size;[8] (2) the complement of this species includes chromosomes large enough to allow a detailed cytological analysis;[9] (3) *R. rubilio* is widespread and abundant in Sicily.

MATERIALS AND METHODS

Rutilus rubilio (Pisces, Cyprinidae) specimens were collected by seine from natural populations inhabiting the artificial basin Arancio Lake,

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Table 1 Genotoxic activity: mitotic metaphase chromosomal damage in *R. rubilio* specimens treated with amox · $3H_2O$, $R_2SnClamox \cdot 2H_2O$ and $R_2Snamox_2 \cdot 2H_2O$ (R = methyl, butyl and phenyl)

Compound	Concn (mol dm^{-3})	Time interval (h)	No. of metaphases					
			Normal	Irregular staining	Granular Zones	Breakages	Sidearm bridges	Total spreads
Control			326	3	2	1	1	333
amox · $3H_2O$	10^{-5}	24	5	12	1	1	—	15
		48	7	17	4	2	3	28
amox · $3H_2O$	10^{-7}	24	8	22	3	—	2	31
		48	10	26	5	3	6	42
$Me_2SnClamox \cdot 2H_2O$	10^{-5}	24	12	10	6	2	3	24
		48	8	6	2	—	3	18
$Me_2SnClamox \cdot 2H_2O$	10^{-7}	24	7	23	8	4	—	36
		48	7	18	7	3	12	40
$Me_2Snamox \cdot 2H_2O$	10^{-5}	24	Died after a treatment of 13–14 h					
		48						
$Me_2Snamox_2 \cdot 2H_2O$	10^{-7}	24	22	6	3	4	2	29
		48	13	8	5	4	4	25
$Bu_2SnClamox \cdot 2H_2O$	10^{-5}	24	1	16	5	3	—	21
		48	2	20	7	4	3	32
$Bu_2SnClamox \cdot 2H_2O$	10^{-7}	24	3	28	12	6	11	51
		48	2	34	16	7	16	66
$Bu_2Snamox \cdot 2H_2O$	10^{-5}	24	Died after a treatment of 2–3 h					
		48						
$Bu_2Snamox_2 \cdot 2H_2O$	10^{-7}	24	—	14	8	—	11	27
		48	1	16	13	6	14	43
$Ph_2SnClamox \cdot 2H_2O$	10^{-5}	24	2	13	—	—	4	17
		48	1	13	14	1	5	29
$Ph_2SnClamox \cdot 2H_2O$	10^{-7}	24	7	23	12	2	—	40
		48	4	25	17	3	—	44
$Ph_2Snamox_2 \cdot 2H_2O$	10^{-5}	24	Died after a treatment of 2–3 h					
		48						
$Ph_2Snamox_2 \cdot 2H_2O$	10^{-7}	24	3	12	3	2	7	24
		48	1	19	16	10	9	48

Sicily, during many different trips to this site.

Specimens were incubated in the presence of light, either in solutions at different concentrations and exposure times with $R_2SnClamox \cdot 2H_2O$ and $R_2Snamox_2 \cdot 2H_2O$ solutions, and for comparison purposes, of amox · $3H_2O$ or in fresh water as controls (Table 1). The parent diorganotin(IV) dichlorides were not tested, since their toxicity was previously evaluated towards freshwater fish *Aphanius fasciatus*[2] and towards the mesogastropod mollusc *Truncatella subcylindrica*.[6]

Amox · $3H_2O$ was a US Biochemical Corporation (Cleveland, OH, USA) product, while $R_2SnClamox \cdot 2H_2O$ and $R_2Snamox_2 \cdot 2H_2O$ have been obtained by previously described procedure.[3] Concentrated stock solutions were obtained by dissolving stoichiometric amounts of each compound in Millipore-filtered fresh water (MFFW). Working solutions (pH 7.8–8.0) were obtained by further dilution of the stocks in MFFW. All diorganotin(IV)–amoxicillin derivatives are stable in polar solvents, as established by previously reported ^1H and ^{13}C NMR data.[3] Organotin(IV) concentrations in the diluted solutions were assayed using a Model 372 Perkin–Elmer graphite furnace atomic absorption spectrophotometer. Chromosome preparations were obtained by the air-drying technique.

Untreated (controls), and treated fish, were injected intraperitoneally with 0.1–0.2 cm^3 of a 0.25% colchicine solution and sacrificed 2 h later. The gills were removed and treated with

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 treated according to the solid tissue technique described by Vitturi.[10]

For conventional analysis of mitotic chromosomes, slides were stained in a 5% Giemsa solution (pH 6.8) for 20 min, rinsed in tap-water and permanently mounted in Canada balsam. Fluorescence banding was performed by staining with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. Slides were washed in running tap-water, dried and mounted in McIlvaine's buffer, pH 7–glycerol (1:1).

Giemsa-stained chromosomes were observed with a Jenamed 2 light microscope and photographed using Agfa Gevaert AG 25 film, while DAPI-stained chromosomes were observed with a Leitz fluorescence microscope and photographed using Kodak Tmax 400 film. Chromosomes were classified according to Levan *et al.*[11]

RESULTS

Since chromosome aberrations can be recognized only after a comparison among the chromosomes of untreated (controls) and treated specimens, the karyotype of the former is first described. It consists of 50 chromosomes which could be arranged in 25 homomorphic pairs (Fig. 1a), eight being metacentric (pairs 1–8), four submetacentric (pairs 9–12), twelve subtelocentric (pairs 13–24) and one acrocentric (pair 25). A few spreads per specimen (2–3%) showed a diploid chromosome number lower than the mode. No spreads possessing extra chromosomes have been encountered.

In all spreads of controls conventionally stained with Giemsa, chromosomes looked like homogeneously and deeply stained bodies with regular outlines (Fig. 1b), except for a low percentage (1–2%) which displayed some anomalies (Table 1). Similarly, after DAPI staining in controls, nearly all chromosomes fluoresced homogeneously (Fig. 1c).

With respect to the controls, in specimens

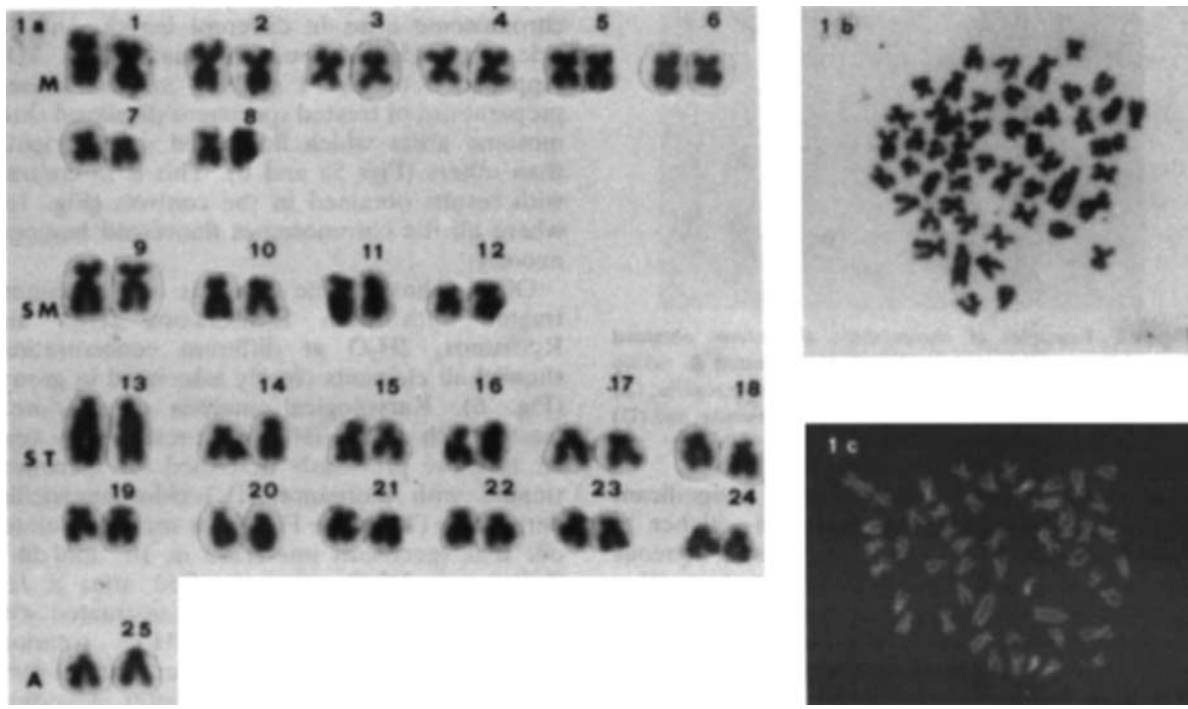


Figure 1 Mitotic metaphase chromosomes of *R. rubilio* controls: (a) karyotype; (b) Giemsa-stained metaphase; (c) DAPI-stained metaphase.

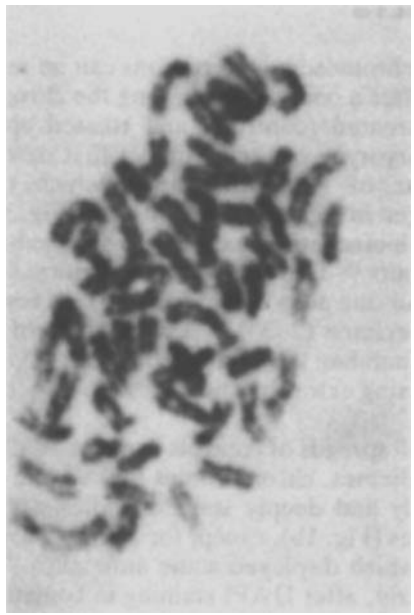


Figure 2 Giemsa-stained metaphase spread of *R. rubilio* treated with 10^{-7} mol dm^{-3} $Me_2SnClamox \cdot 2H_2O$ for 24 h.

Figure 4 Giemsa-stained metaphase spread of *R. rubilio* treated with 10^{-7} mol dm^{-3} $Bu_2SnClamox \cdot 2H_2O$ for 48 h.

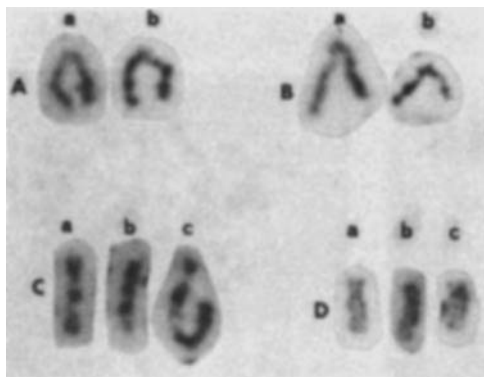
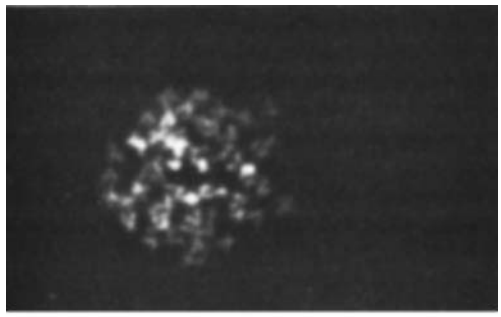


Figure 3 Examples of chromosome aberrations obtained from different Giemsa-stained spreads of treated *R. rubilio* specimens: (A) chromosomes with black granular regions; (B) breakages; (C) chromosomes with irregular staining; and (D) chromosomes with pseudochiasmata.

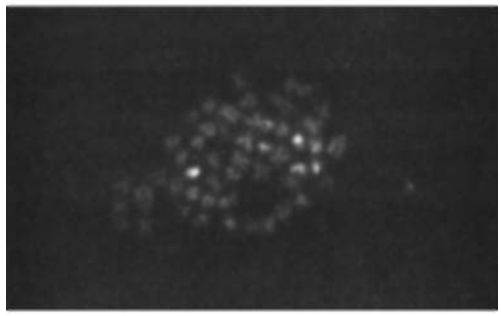
treated with amoxicillin derivatives a significant increase of chromosome anomalies—higher in specimens treated with less concentrated aqueous solutions (10^{-7} mol dm^{-3})—has been observed.

Chromosome aberrations, listed in Table 1, include: (1) differentially stained areas (Fig. 2) which conferred upon the chromosomes a banded appearance (Fig. 3C); (2) granular deeply stained zones terminally (Fig. 4) and/or interstially located (Fig. 3A; see b); (3) arm breakages (Fig. 3B), mainly suggested by the presence of chromosome arms in different length, and (4) side-arm bridges (pseudochiasmata) (Fig. 3D). Application of DAPI staining in chromosome preparations of treated specimens displayed chromosome areas which fluoresced more brightly than others (Figs 5a and b). This is in contrast with results obtained in the controls (Fig. 1c), where all the chromosomes fluoresced homogeneously.

Often chromosome spreads of specimens treated with both $R_2SnClamox \cdot 2H_2O$ and $R_2Snamox_2 \cdot 2H_2O$ at different concentrations showed all elements closely associated in groups (Fig. 6). Karyological analysis of specimens treated with $amox \cdot 3H_2O$ gave results very similar to those previously described for specimens treated with diorganotin(IV)–chloroamoxicillin derivatives (Table 1). Finally, it must be pointed out that specimens immersed in 10^{-5} mol dm^{-3} $R_2Snamox_2 \cdot 2H_2O$ solutions died after a few hours' treatment, while specimens treated with 10^{-5} mol dm^{-3} $R_2SnClAmox \cdot 2H_2O$ solutions survived more than 48 h. However, chromosome preparations obtained from the latter individuals showed a small number either of interphase nuclei or of metaphase chromosome spreads.



(a)



(b)

Figure 5 (a) and (b) DAPI-stained metaphase spreads of *R. rubilo* treated with 10^{-5} mol dm^{-3} $Me_2SnClAmox \cdot 2H_2O$ for 48 h. More condensed zones fluoresce more brightly than others.

DISCUSSION

Two different chromosome-staining techniques—one using Giemsa and the other the fluorochrome DAPI—have successfully been employed for the analysis of mitotic metaphases of untreated specimens (controls) of *Rutilus rubilio* and specimens treated with the semisynthetic antibiotic amox · $3H_2O$ and with $R_2SnClamox \cdot 2H_2O$ and $R_2Snamox_2 \cdot 2H_2O$ ($amox^- = 6$-[D(−)-β-amino-*p*-hydroxyphenylacetamido]penicillinate) (R = methyl, butyl and phenyl). As previously observed in *Aphanius fasciatus* controls,[6] in *R. rubilio* controls as well, a few cells showed a hypodiploid chromosome number.

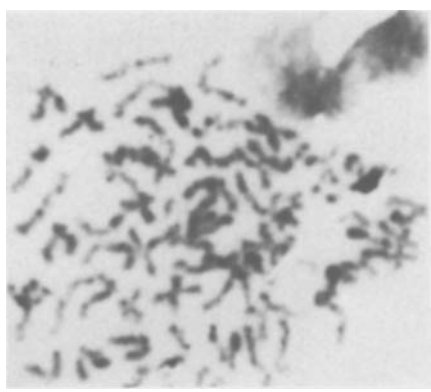


Figure 6 Giemsa-stained metaphase spread of *R. rubilio* treated with 10^{-7} mol dm^{-3} $Me_2SnClamox \cdot 2H_2O$ for 24 h. More than one anomaly is detectable.

Due to the presence of a nearly identical number of aneuploid cells in both treated and untreated (controls) *R. rubilio* specimens, the occurrence of aneuploidy is presumably to be attributed to technical shortcomings, rather than to any real action of chemicals used in this study. This conclusion is mainly supported by the absence of hyperdiploid spreads which, according to Dean and Danford,[12] provides a conclusive proof of the occurrence of this phenomenon. Moreover, since in *R. rubilio* controls, other cells [although in a very low percentage (1–2%)] displayed chromosome abnormalities such as breakages, side-arm bridges and/or faintly stained chromosomes, a spontaneous background of chromosome anomalies might occur in their karyotype.

Data, summarized in Table 1, suggest some considerations: (1) in agreement with previous results,[1] methyl derivatives seem to exert a lower cytotoxicity than that exerted by butyl and phenyl analogues. This conclusion is reliably supported by the fact that individuals treated with 10^{-5} mol dm^{-3} $R_2Snamox_2 \cdot 2H_2O$ solutions (R = butyl and phenyl) died a few hours after the beginning of the treatment, while individuals immersed in $Me_2Snamox_2 \cdot 2H_2O$ at the same concentration survived 13–14 h; (2) $R_2Snamox_2 \cdot 2H_2O$ derivatives are more toxic than $R_2SnClamox \cdot 2H_2O$ at both 10^{-5} and 10^{-7} mol dm^{-3} concentrations; and (3) specimens treated with 10^{-5} mol dm^{-3} $R_2SnClamox \cdot 2H_2O$ (R = methyl, butyl and phenyl) solutions showed a lower number of either interphase nuclei or cleaving nuclei in the form of chromosome spreads than specimens treated with the same solutions at 10^{-7} mol dm^{-3} concentration. A reliable explanation of the latter point might be that cytotoxicity of 10^{-5} mol dm^{-3} solutions is so high that it produces irreparable damage to the process of cell division.

As reported in a previous paper for *A. fasciatus*,[6] in *R. rubilio* also the most frequent observed chromosome anomaly is the presence of gaps or 'achromatic lesions', which give to the chromosome a banded appearance. Chromosome

banded appearances are clearly visualized only when chromosomes display a thread-like morphology. This would indicate that such an anomaly might be linked to a different degree of DNA condensation which is more readily detectable when chromosomes are more despiralized. Moreover, this notion is consistent with the occurrence along the chromosomal body of over-condensed zones resulting in deeply stained areas after Giemsa staining, as well as chromosome zones which fluoresced more brightly than others after DAPI staining.

With regard to the latter point, it is widely admitted that a higher fluorescence is shown by those chromosome portions which consist of (A + T)-rich DNA and/or more condensed than others. Since in *R. rubilio* controls all chromosomes fluoresced homogeneously, thus suggesting that (A + T)-rich and (G + C)-rich DNAs are equally interspersed in the karyotype. The notion that a higher fluorescence may be due to a (A + T)-rich DNA portion can be discharged.

Another interesting observation can be drawn from results of this research: amox · $3H_2O$ is as toxic as is derivatives. This finding disagrees with results obtained using another semisynthetic antibiotic, penicillin G (penG), which seemed to exert no significant toxic activity on chromosomes of *Aphanius fasciatus* (Pisces, Cyprinodontiformes).[2] The diversification of responses to these semisynthetic antibiotics might be found in the difference in chemical composition between penG and amox.[1,3]

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REFERENCES

1. F. Maggio, A. Pellerito, L. Pellerito, S. Grimaudo, C. Mansueto and R. Vitturi, *Appl. Organomet. Chem.* **8**, 71 (1994).
2. R. Vitturi, C. Mansueto, A. Gianguzza, F. Maggio, A. Pelleruto and L. Pellerito, *Appl. Organomet. Chem.* **8**, 509 (1994).
3. L. Pellerito, F. Maggio, M. Consiglio, A. Pellerito, G. C. Stocco and S. Grimaudo, *Appl. Organometal. Chem.* **9**, 227 (1995).
4. C. Mansueto, M. Gianguzza, G. Dolcemascolo and L. Pellerito, *Appl. Organomet. Chem.* **7**, 391 (1993).
5. C. Mansueto, L. Pellerito, M. A. Girasolo and M. Lo Valvo, *Appl. Organomet. Chem.* **7**, 95 (1993).
6. R. Vitturi, C. Mansueto, E. Catalano, M. A. Girasolo and L. Pellerito, *Appl. Organomet. Chem.* **6**, 525 (1992).
7. R. Vitturi, L. Pellerito, E. Catalano and M. R. Lo Conte, *Appl. Organomet. Chem.* **7**, 295 (1993).
8. E. Tortonese, *Fauna d'Italia*, Vol. X, *Osteichthyes*, Edizioni Calderini, Bologna, 1970.
9. S. Catudella, L. Sola, R. Accame Muratori and E. Capanna, *Genetica* **47**, 161 (1977).
10. R. Vitturi, *Chromatin* **1**, 147 (1992).
11. A. Levan, K. Fredga and A. Sandberg, *Hereditas* **52**, 201 (1964).
12. B. J. Dean and N. Danford, Assays for the detection of chemically induced chromosome damage in cultured mammalian cells. In: *Mutagenity Testing: A Practical Approach*, Vermit, S. and Parry, J. M. (eds), Oxford University Press, Oxford, 1984, pp. 187–232.