Biochemical Pharmacology, Vol. 36, No. 9, pp. 1555–1559, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

DIFFERENT EFFECTS OF OXYTETRACYCLINE AND DOXYCYCLINE ON MITOCHONDRIAL PROTEIN SYNTHESIS IN RAT LIVER AFTER LONG-TERM TREATMENT

COBY VAN DEN BOGERT,* MARIJKE HOLTROP, TRUDI E. MELIS, PIETER R. ROEFSEMA and ALBERT M. KROON

Laboratory of Physiological Chemistry, State University Medical School, Bloemsingel 10, 9712 KZ Groningen, The Netherlands

(*Received* 21 *August* 1986; *accepted* 5 *December* 1986)

Abstract—The tetracyclines inhibit specifically mitochondrial (mt) and bacterial protein synthesis when they are present in low concentrations (2–10 μg/ml). There is no difference between the various members of this group of antibiotics in this respect. In the present study, however, it is shown that the inhibitory effect of doxycycline on mt-protein synthesis in rat liver is partially lost after continuous treatment for more than 1 week, whereas oxytetracycline continues to inhibit mt-protein synthesis effectively after 1 week of treatment.

To find an explanation for this difference between doxycycline and oxytetracycline, a detailed study was made of the distribution and the effects on mt-protein synthesis of both tetracyclines under various conditions in rat liver. The results of the studies lead to the hypothesis that doxycycline treatment induces the formation of a doxycycline complex, and thus to a reduced amount of free doxycycline. This may explain the loss of effective inhibition of mt-protein synthesis.

It has been shown before that the growth of several tumor systems in the rat becomes arrested as a secondary effect of specific inhibition of mitochondrial (mt-)protein synthesis [1–4].

In such studies, we employ the tetracyclines to inhibit mt-protein synthesis and to investigate the possible cytostatic effect thereof. Due to the similar sensitivity of ribosomes from mitochondria and bacteria to these antibiotics, the tetracyclines block at the same low concentration bacterial as well as mt-protein synthesis [5]. Initially, mainly oxytetracycline (OTC) was used to inhibit mt-protein synthesis. Later, we employed also doxycycline (DC), because the latter member of the tetracycline group possesses favourable pharmacokinetics for clinical studies on the cytostatic action of the tetracyclines in man [6]. In experiments on the antitumor effect of tetracycline treatment in animal systems, no differences between OTC and DC were found. Only in normal rat liver, however, DC appears to inhibit mt-protein synthesis during continuous treatment far less effectively than OTC. The nature and possible cause of the differences between both tetracyclines are reported in the present study.

MATERIAL AND METHODS

Animals and tetracyclines. Male Wistar rats, weighing about 250 g, were treated with tetracyclines by means of continuous i.v. infusion as described previously [5]. Oxytetracycline (OTC)—sub forma HCl—and doxycycline (DC)—in its standard commercial preparation form for i.v. injection (Vibramycin®)—were generous gifts from Gist-Brocades N.V. (Delft, The Netherlands) and Pfizer B.V. (Rotterdam, The Netherlands), respectively.

Collection of samples. At the end of each experiment blood was aspired from the vena cava inferior. Serum was obtained by centrifugation of the blood samples. The liver was removed and a 10% homogenate was made in cold 0.25 M sucrose with the aid of a Teflon pestle–glass homogenizer. Liver mitochondria were prepared according to the method of Hogeboom [7].

Aminoacid incorporation by isolated liver mitochondria. The aminoacid incorporation activity of freshly prepared mitochondria was measured at 30°. The medium contained 50 μmoles tricine buffer, 20 μmoles KCl, 30 μmoles NH_4Cl, 7.5 μmoles $MgCl_2$, 1 μmole EDTA, 30 μmoles sodium succinate, 2 μmoles ADP, 20 μmoles potassium phosphate, 50 μg cycloheximide, 0.76 μmoles of an aminoacid mixture not containing leucine, 50 μmoles sucrose, 3 mg mitochondrial protein and tetracyclines as indicated in the experiments. The final pH was 7.4, the final volume 1 ml. After a 5-min period of preincubation the reactions were started by the addition of 0.04 μmole [^{14}C]leucine (10 μCi/μmole). After incubation for 45 min the reactions were terminated by the addition of 10 μmoles unlabelled leucine and 5 ml 5% TCA. The precipitates were collected and defatted, whereafter radioactivity was counted in a liquid scintillation counter.

Analytical methods. Cytochrome *c* oxidase activity was assayed spectrophotometrically [8]. The activity was calculated per min per mg protein and expressed as the first-order reaction rate constant, *k*. The amount of cytochrome pigments was calculated from difference spectra. The extinction coefficients and wavelength pairs as described by Estabrook and

* To whom correspondence should be addressed.

Holowinsky [9] were used to calculate the cytochrome concentrations.

Protein was determined with a modified Lowry method [10]. The OTC or DC contents of sera, homogenates and mitochondria were assayed fluorometrically [11] as well as by a HPLC method [12].

RESULTS AND DISCUSSION

Continuous inhibition of mt-protein synthesis in mammalian cells leads to a gradual reduction of the cellular content of mt-gene products. The polypeptides which are encoded by mt-DNA are all part of enzymes involved in oxidative phosphorylation [13]. If mt-protein synthesis is inhibited, the specific activity of these enzymes will decrease until the oxidative phosphorylation capacity of the cell has been reduced to an extent that cellular processes like cytoplasmic protein synthesis become hampered. Then the specific activities of the partly mt-made enzymes will not decline further, because, secondary to inhibition of mt-protein synthesis, also the total amount of protein is reduced.

Cytochrome *c* oxidase or its spectral equivalent cytochrome aa_3 is one of the components of the oxidative phosphorylation system which consists partly of subunits of mt-genetic origin. The enzyme is located exclusively in mitochondria. If mt-protein synthesis is inhibited selectively the specific cytochrome *c* oxidase activity of either homogenates or mitochondria as well as the mt-cytochrome aa_3 content are reliable measures for the effect of *in vivo* tetracycline treatment on mt-protein synthesis.

Figure 1 shows that DC does not reduce the specific cytochrome *c* oxidase activity in liver mitochondria if rats have been treated for periods longer than about 1 week, whereas OTC continues to do this also after 1 week of constant administration. A comparable lack of effective reduction by DC was found by measuring the cytochrome *c* oxidase activity in liver homogenates. This implies that the different effect of DC and OTC is not based on differences in the composition of mitochondria prepared from livers of either DC- or OTC-treated rats. Moreover, the yield of mitochondria from livers of control or tetracycline-treated rats was well comparable. Therefore, it can be concluded that DC, in contrast to OTC, does not effectively impair mt-protein synthesis in the liver of rats which have been treated with DC for more than one week.

There are a number of possible explanations for the difference between the two tetracyclines. First, it might be that the intracellular or intramitochondrial DC content changes during prolonged treatment. The ratio of the tetracycline concentration in the liver homogenate as well as of the tetracycline concentration in liver mitochondria to the serum level was therefore investigated after prolonged treatment (Table 1). It can be concluded that, as discussed in a previous paper [5], the distribution for OTC and DC is not the same. This is based on their dissimilar pharmacokinetic properties, due to differences in serum-protein binding and lipid solubility. These dissimilarities in distribution are not only reflected in a 3-fold higher homogenate to serum

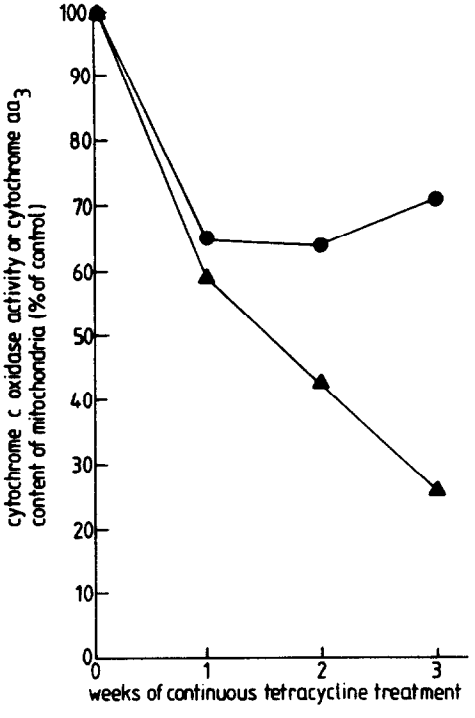

Fig. 1. Effect of continuous treatment with DC or OTC on the specific cytochrome *c* oxidase activity or cytochrome aa_3 content of liver mitochondria after different periods of treatment. Rats were treated by means of continuous infusion with 10–60 mg DC/kg/day or 20–120 mg OTC/kg/day, resulting in serum levels between 2 and 10 μg/ml. The mean values of minimal 15 experiments per point are given, the SE was maximally 2.5% of the mean value: ●, DC treated; ▲, OTC treated; 100% cytochrome *c* oxidase activity, $K = 27.6 \pm 0.84$ (SE)/mg mt-protein (N = 32); 100% cytochrome aa_3, 152.1 pmol ± 3.2 (SE)/mg mt-protein (N = 51).

ratio for DC, but also in a significantly higher mitochondria to homogenate ratio. For DC the latter ratio is about 2.4, whereas it is 1.8 for OTC.

A relationship between the duration of treatment and the intracellular or mitochondrial DC or OTC content is, however, clearly absent. Continuous infusion makes it possible to maintain fairly constant serum levels [5]. Significant changes of the tetracycline content of the homogenate or the mitochondria during prolonged treatment are thus excluded.

Second, it might be that DC, because of its higher intracellular level, inhibits directly also cytoplasmic protein synthesis. If this were the case, then the *specific* cytochrome *c* oxidase activity would not decline further because total cellular protein synthesis is impaired, which masks the effect of specific inhibition of mt-protein synthesis. Inhibition of cytoplasmic protein synthesis by DC is, however, unlikely because the lack of specific inhibition of mt-protein synthesis after 1 week of treatment is not accompanied by increasing DC tissue levels. Moreover, liver regeneration after partial hepatectomy, for which cytoplasmic protein synthesis is a prerequisite, was unimpaired up to 3 weeks of DC treatment (data not shown).

Table 1. Tissue distribution of DC or OTC after different periods of continuous treatment at serum levels of 4–10 μg DC or OTC per ml

Period of treatment (weeks)	Tetracycline	μg/mg protein / μg/ml serum	
		Homogenate	Mitochondria
1	OTC	0.028 ± 0.01 (19)	0.051 ± 0.02 (19)
2	OTC	0.036 ± 0.02 (18)	0.065 ± 0.03 (18)
3	OTC	0.034 ± 0.01 (11)	0.063 ± 0.03 (11)
1	DC	0.093 ± 0.02 (20)	0.229 ± 0.05 (20)
2	DC	0.091 ± 0.03 (19)	0.221 ± 0.05 (19)
3	DC	0.100 ± 0.03 (10)	0.241 ± 0.06 (10)

The rats were treated with 20–60 mg DC/kg/day or with 40 to 120 mg OTC/kg/day. Tissue distribution is expressed as the ratio between the tissue concentration (μg/mg protein) and the serum concentration (μg/ml). The number of experiments is indicated between the brackets, the mean ratio ± SE is given.

Third, DC may slow down the turnover rate of mitochondria after prolonged treatment. If so, then DC should be able to inhibit mt-protein synthesis during liver regeneration because under these conditions proliferation is required. However, mt-protein synthesis was not effectively impaired during liver-cell proliferation after partial hepatectomy when the rats had been treated before partial hepatectomy for more than 3 days with DC.

Fourth, DC might inhibit mt-protein synthesis less effectively in the liver because prolonged treatment induces resistance of mt-ribosomes to inhibition by DC.

To test this possibility, the capacity of control mitochondria to incorporate aminoacids *in vitro* was compared to that of liver mitochondria from rats treated for 1 or 14 days with OTC or DC. Moreover, the effect of adding various amounts of tetracyclines on protein synthesis in isolated mitochondria was studied.

Figure 2 shows the effect of OTC or DC on the aminoacid incorporation activity of isolated mitochondria from control livers. The incorporation activity of the various preparations, expressed as pmoles per unit of protein, shows rather large variations. The percentage of inhibition of mt-protein synthesis by a given amount of tetracyclines ranges, however, within narrow limits (circa 5%), which allows the construction of the mean control curves as given in Fig. 2. The amount of tetracycline added is plotted against the percentage of incorporation activity. Parallel experiments with unlabeled leucine were carried out to determine the tetracycline concentration of the mitochondria during the aminoacid incorporation studies. At the concentrations tested the ratio between the tetracycline concentration in the aminoacid incorporation medium and the mitochondria was constant. For OTC this ratio was 0.9, for DC 0.5. Together with the data of Fig. 2, these ratios allow a direct comparison between the amino acid incorporation activity of the various mt preparations. Addition of 1 μg tetracycline to the medium equals 0.1 μg OTC/mg mt-protein and 0.5 μg DC/mg mt-protein. Data on mitochondria which contain already tretracyclines due to *in vivo* treatment can also be analyzed, since 1 μg tetracycline per mg mt-protein of mitochondria to which no tetracyclines are added *in vitro* corresponds to 10 μg OTC and 2 μg DC per mg mt-protein in case these tetracyclines were added to control mitochondria. The aminoacid incorporation activity of the various mitochondria can thus be extrapolated to a zero tetracycline content. Also the percentage of inhibition of the incorporation activity of pretreated mitochondria

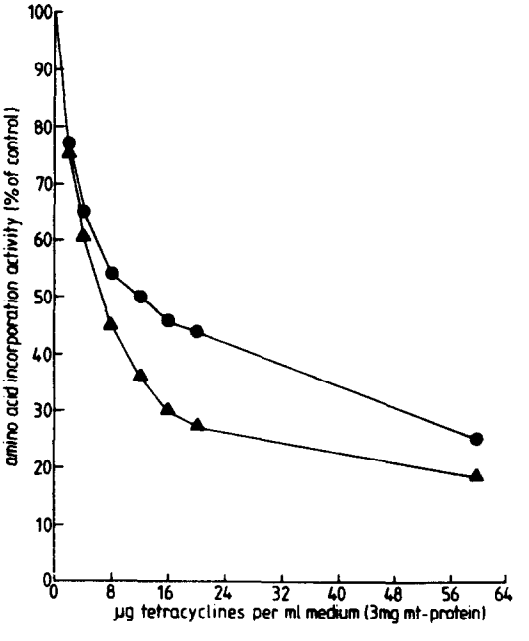

Fig. 2. The effect of added DC or OTC on the aminoacid incorporation activity of mitochondria isolated from control livers. The aminoacid incorporation activity was measured as the amount of incorporated [^{14}C]leu. The control incorporation activity ranges between 32–64 pmoles of [^{14}C]leu incorporated during 45 min per mg mt-protein. The mean percentage inhibition of mt-protein synthesis found in 3 controls is given; individual values ranged no more than 5% of the mean: ●, DC; ▲, OTC.

Table 2. Effect of tetracyclines on the aminoacid incorporation activity of mitochondria isolated from livers of control or tetracycline treated rats

Kind of treatment; *in vivo; in vitro*	Tetracycline concentration (μg/ml medium)			pmol[^{14}C] leu incorporated per mg protein
	Due to *in vivo* treatment	Due to *in vitro* addition	Total concentration	pmol; percentage
Control; —	0	0	0	36–64; 100
Control; OTC	0	11	11	40
Control; DC	0	28	28	40
1 day OTC; —	0*	0	0*	39–60; 100
1 day OTC; —	4	0	4	60
1 day OTC; OTC	4	8	12	40
1 day OTC; DC	4	25	29	40
1 day DC; —	0*	0	0*	28–49; 100
1 day DC; —	4	0	4	70
1 day DC; OTC	4	8	12	40
1 day DC; DC	4	40	44	40
14 days OTC; —	0*	0	0*	39–67; 100
14 days OTC; —	4	0	4	65
14 days OTC; OTC	4	7	11	40
14 days OTC; DC	4	26	30	40
14 days DC; —	0*	0	0*	73–108; 100
14 days DC; —	4	0	4	60
14 days DC; OTC	4	8	12	40
14 days DC; DC	4	>72	>76	>40

Rats were treated with tetracyclines as described in the legend of Fig. 1.

Mitochondria were isolated and their aminoacid incorporation activity tested, with or without the addition of tetracyclines. The tetracycline content of the mitochondria was also assayed. The DC or OTC content of the mitochondria is expressed as μg TC/ml incubation medium to allow an easy comparison of the data. 1 μg TC/ml medium equals 0.03 μg OTC/mg mt-protein and 0.17 μg DC/mg mt-protein (see text).

* 100% extrapolated to a zero tetracycline concentration. The mean values of at least 3 experiments per experimental group with mitochondria which contained 4 μg TC/ml due to *in vivo* treatment are given. The ranges of the absolute incorporation activity are given for at least 6 experiments per experimental group.

by the addition of tetracyclines *in vitro* can be compared with that of control mitochondria by determining the percentage of inhibition at the same total mt-tetracycline content (added + present vs added). Table 2 shows some of the results of these studies.

From this table the following conclusions can be drawn. It requires a higher DC than OTC concentration to inhibit mt-protein synthesis to the same extent. This is partly explained by the fact that OTC is readily lost from the mt-preparations. About 40% of the mt-associated OTC is lost during centrifugation and washing steps carried out after the aminoacid incorporation studies, whereas no DC is lost. It can thus be concluded that mitochondria contain 3 times more DC than OTC if the same amount of tetracyclines is added. This results in a comparable inhibition of mt-protein synthesis when 0–4 μg of the tetracyclines are added. We assume that more DC than OTC is present in a bound form and that the amount of free antibiotic is similar under these conditions. At higher amounts of DC (Fig. 2) even more than 3 times the amount of OTC is needed to obtain the same degree of inhibition by DC, suggesting that the amount of free DC becomes relatively less at higher concentrations.

It can also be seen in Table 2 that there are no significant differences between mitochondria from control or OTC pretreated rats. The extrapolated 100% incorporation activity is in the same range as is the amount of tetracyclines needed to inhibit protein synthesis to 40%. In rats, pretreated with DC for 1 day, the amount of DC which has to be added to achieve 60% inhibition is about 1.5 times higher than in mitochondria of control or OTC pretreated rats. In rats pretreated for 14 days with DC, 60% inhibition could not be reached, mt-protein synthesis seems more or less insensitive to inhibition by DC. Moreover, the extrapolated 100% incorporation activity is about twice that in the controls, suggesting that mt-protein synthesis is also *in vivo* hardly affected any more by DC. *In vitro* mt-protein synthesis is, however, sensitive to OTC irrespective of pretreatment of the rats with DC for 1 day or 2 weeks. Since the mechanism by which the various tetracyclines inhibit mt- and bacterial protein synthesis is presumably the same, it is thus unlikely that prolonged DC treatment induces resistance of mt-ribosomes to DC.

Finally, it may be that the inhibitory effect of DC is lost after long-term treatment because of the induction of mechanisms which cause either transformation of DC to an inactive metabolite or binding to, or influx of, some factor which results in the formation of an inactive DC complex. The difference

between free and complexed DC is not measurable under our experimental conditions. Induction of mechanisms which lead to biotransformation of DC is not a probable explanation, because only the active form of DC is measured in the fluorometric assay [11] and the mitochondrial DC content does not change during prolonged treatment, whereas the effect on mt-protein synthesis vanishes. Degradation products were also not detectable by HPLC analysis.

By deduction, we favor the following explanation. The uptake of DC by mitochondria is accompanied, or followed, by uptake of some other compound, and DC and this compound subsequently form a complex in which DC is not able to inhibit mt-protein synthesis. The amount of the compound the uptake of which is induced by DC is non-linearly related to the mitochondrial DC concentration: high DC concentrations lead to a relatively higher influx than low concentrations. At high mitochondrial DC levels or after prolonged DC treatment only a small fraction of the total amount of DC will be present in the free form, resulting in a decreased inhibition of mt-protein synthesis. The identity of this postulated compound is unknown. However, it should also be present in the incubation medium used during the *in vitro* studies, since *in vitro* the inhibitory effect of DC on mt-protein synthesis is also counteracted at higher DC levels. The OTC complexing properties of the compound concerned are either much less or the influx of the compound is reduced in the case of OTC treatment, because of the lower mitochondrial OTC concentration. The identity of the unknown compound is presently under investigation.

Irrespective of the nature of the ineffectiveness of prolonged DC treatment on mt-protein synthesis in the liver, the difference between DC and OTC is of interest for two major reasons. First, the clinical use of DC in (antibacterial) treatment is generally preferred to that of other tetracyclines, because of its pharmacokinetic properties. The lipophilicity and strong binding to serum proteins of DC result in a prolonged serum half time as compared to the other tetracyclines [6]. Moreover, the rate of excretion of DC is not dependent on renal function. DC can, therefore, also be used in the case of renal insufficiency. Interestingly, side effects on liver functions are less frequently found for DC than for other tetracyclines. In our animal studies, the tetracycline serum levels are in the same range as those achieved in antibacterial treatment of man. The lack of effective inhibition of mt-protein synthesis by DC may, if extrapolation is allowed, account for the reduced frequency of side effects of DC on liver functions in man. Second, it may turn out that DC inhibits mt-protein synthesis also in some tumor types less effectively than for instance OTC. If this is true, then DC should be replaced by another tetracycline in clinical studies with these tumor types, despite the favorable pharmacokinetic properties of DC in man.

Acknowledgements—This study was supported in part by a grant of the Dutch Foundation for Cancer Research, The Koningin Wilhelmina Fonds. The authors wish to thank Gist-Brocades N.V., Delft, The Netherlands, and Pfizer B.V., Rotterdam, The Netherlands, for their gifts of OTC and DC respectively, Mr E. H. J. Dontje and Mr J. J. Wijbenga for their technical assistance and Mrs R. Kuperus for typing the manuscript.

REFERENCES

1. C. van den Bogert, B. H. J. Dontje, J. J. Wijbenga and A. M. Kroon, *Cancer Res.* **41**, 1943 (1981).
2. C. van den Bogert, B. H. J, Dontje and A. M. Kroon, *Cancer Res.* **43**, 2247 (1983).
3. C. van den Bogert, B. H. J. Dontje and A. M. Kroon, *Leukemia Res.* **9**, 617 (1985).
4. C. van den Bogert, B. H. J. Dontje, M. Holtrop, T. E. Melis, J. C. Romijn, J. W. van Dongen and A. M. Kroon, *Cancer Res.* **46**, 3283 (1986).
5. C. van den Bogert and A. M. Kroon, *Biochem. Pharmac.* **30**, 1706 (1981).
6. A. M. Kroon and C. van den Bogert, *Pharm. Weekbl. (Sci.)* **5**, 81 (1983).
7. G. H. Hogeboom, *Meth. Enzymol.* **1**, 16 (1955).
8. P. Borst, G. J. C. M. Ruttenberg and A. M. Kroon, *Biochim. biophys. Acta* **149**, 140 (1967).
9. P. W. Estabrook and A. Holowinsky, *J. biophys. biochem. Cytol.* **9**, 19 (1961).
10. G. L. Peterson, *Analyt. Biochem.* **83**, 346 (1977).
11. C. van den Bogert and A. M. Kroon, *J. Pharm. Sci.* **70**, 186 (1981).
12. R. Böcker, *J. Chromatogr.* **274**, 255 (1983).
13. A. Chomyn, P. Mariottini, M. W. J. Cleeter, C. I. Ragan, A. Matsuno-Yagi, Y. Hatefi, R. F. Doolittle and G. Attardi, *Nature, Lond.* **314**, 592 (1985).