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EFFECTS OF TICLOPIDINE, A NEW PLATELET ANTIAGGREGATING AGENT, AND ITS ANALOGUES ON MITOCHONDRIAL METABOLISM

OXIDATIVE PHOSPHORYLATION, PROTEIN SYNTHESIS AND DNA POLYMERASE ACTIVITY

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Abstract—The effects of ticlopidine and six of its analogues on mitochondrial functions were studied in isolated rat liver mitochondria. The influence of ticlopidine and each of the following analogues: PCR 5325, PCR 4099, PCR 3787, PCR 2362, PCR 4499 and PCR 0665 was evaluated by determining their interaction with three major mitochondrial activities. (A) Oxidative phosphorylation, measured by oxypolarography, was assayed in the presence of glutamate or succinate as source of energy, and both State 4 and State 3 were recorded. Ticlopidine, at 20 μg/ml, slightly increased glutamate State 4, whereas it was without effect on that of succinate. At higher concentration (40 μg/ml), ticlopidine caused 40–45% inhibition of State 4 with both substrates. All the other analogues tested at either 20 or 40 μg/ml were virtually without effect on the respiration. However, at 20 μg/ml, ticlopidine and some of its analogues inhibited mitochondrial State 3, while under similar conditions other analogues had little or no effect on this state. (B) Mitochondrial protein synthesis, measured by [^{14}C]-L-leucine incorporation, was not affected significantly by any of these drugs. Whereas chloramphenicol at 10 μg/ml caused 80% inhibition, ticlopidine and its analogues in concentrations inhibitory to State 3 did not inhibit mitochondrial protein synthesis. (C) Mitochondrial DNA polymerase activity, determined by [^{3}H] thymidine 5′-triphosphate incorporation, was not inhibited by these drugs. We conclude that, while ticlopidine and analogues have little or no effect on either mitochondrial protein synthesis or mitochondrial DNA polymerase activity, ticlopidine and some of its analogues are inhibitory of the energy conserving mechanism in mitochondria.

Ticlopidine, a new inhibitor of platelet aggregation, is used clinically in Europe, Japan and Latin America to reduce morbidity and mortality in patients with atherosclerosis. Its use has occasionally been associated with severe granulocytopenia [1, 2]. The pathogenesis of this complication remains uncertain. Recent studies in our laboratory† have demonstrated that ticlopidine and some of its analogues inhibit granulopoiesis *in vitro*, suggesting a direct toxic cellular effect from these drugs.

Little is known about ticlopidine interaction with subcellular organelles. Mitochondria, power house of the cell and major site of cellular metabolism, have been chosen in the present study as a possible target for ticlopidine toxicity. Three major mitochondrial activities, oxidative phosphorylation, protein synthesis and DNA synthesis, were tested *in vitro* in the presence of ticlopidine and six of its analogues (cf. Fig. 1). Our results showed that ticlopidine and some of its analogues may be potent inhibitors of oxidative phosphorylation by interfering somehow with the energy conserving mechanism in mitochondria. However, they were without effect on either protein or DNA synthesis by these organelles. Part of this work has been presented.‡

MATERIALS AND METHODS

Preparation of mitochondria. Mitochondria were prepared as described previously [3] from the liver of 5- to 8-week-old Sprague–Dawley male rats fasted overnight. Briefly, the liver was homogenized in a medium consisting of 250 mM sucrose, 10 mM Tris–HCl, 2 mM EDTA, pH 7.4. The homogenate was centrifuged at 800 *g* for 10 min and the supernatant fraction was collected and recentrifuged at 8500 *g* for 12 min. The resulting pellet was washed and recentrifuged twice to obtain the purified mitochondrial pellet.

Respiration and oxidative phosphorylation. This mitochondrial activity was determined by oxypolarography in a double-jacketed cell with a chamber of 2 ml capacity at 30°. A Clark oxygen electrode

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† A. A. Yunis, Abstracts of Symposium "Ticlopidine: Quo Vadis?", Groupe Sanofi – Centre de Recherche Clin-Midy, Montpellier, France, October 20 and 21, p. 13 (1983).

‡ S. Abou-Khalil, W. H. Abou-Khalil and A. A. Yunis, Abstracts of Symposium "Ticlopidine: Quo Vadis?", Groupe Sanofi – Centre de Recherche Clin-Midy, Montpellier, France, October 20 and 21, p. 26 (1983).

and an oxygen monitor (Yellow Springs Instruments Co.) were used. The incubations were carried out in a 2-ml reaction medium consisting of 25 mM glycylglycine, 60 mM KCl, 87 mM sucrose, and 5 mM phosphate adjusted to pH 7.4 with KOH. Metabolic States 4 and 3 were determined according to Ref. 4. The solubility of oxygen in the air-saturated reaction medium was considered to be 445 ng atoms/ml at 30°. The respiratory control ratio of the mitochondrial preparations ranged between 4 and 5. Due to the slight different effects of the drugs on State 4, the percent inhibition of respiratory State 3 was calculated by comparing State 3 relative rate (respiratory rate of recorded State 3 − State 4) to that of the normal control.

Mitochondrial protein synthesis. The assays were carried out in an incubation medium consisting of 50 mM bicine (pH 7.4), 15 mM P_i, 10 mM succinate, 1 mM EDTA, 50 mM KCl, 5 mM nicotinamide, 5 mM ATP, 6 mM $MgCl_2$, 50 units penicillin G/ml, complete amino acid mixture minus leucine as described in Ref. 5, and with or without the drug studied, incubated in a Dubnoff metabolic shaker at 30°. Mitochondria (0.113 mg protein) were added to a total volume of 0.225 ml of the incubation medium, followed 2 min later by the addition of 1.7 μCi [^{14}C]-L-leucine (sp. act. 342 mCi/mmole). At 60 min after leucine addition, the reaction was stopped by applying 0.1-ml aliquots on a 2.3 cm Whatman No. 3 filter paper disk as fully described in Ref. 6, and then the radioactivity was assayed in a liquid scintillation counter. The inhibition of [^{14}C]leucine incorporation in the presence of 200 μg/ml of cycloheximide due to microsomal contamination constituted a background and was subtracted from the respective assays. All materials used were sterilized.

Mitochondrial DNA polymerase activity. Mitochondria were subjected to hypotonic swelling for 10 min in a medium (10 mg protein/ml) containing 30 mM Tris (pH 8.0), 30 mM P_i, 100 μg/ml of bovine serum albumin, and 3 mM β-mercaptoethanol at ice temperature. They were then sonicated twice for 20 sec (at 1-min interval) at 90% intensity in a Fisher Sonic Dismembrator model 300. The sonicated suspension was immediately centrifuged at 100,000 *g* for 45 min in a refrigerated Spinco model L ultracentrifuge, and the supernatant fraction containing the mitochondrial DNA polymerase suspension was used for assays.

This was carried out by determining the incorporation of [^{3}H]TTP into calf thymus DNA which had been activated according to Aposhian and Kornberg [7]. The reaction was started by the addition of mitochondrial DNA polymerase suspension (0.12 mg protein) to a mixture, at 37°, consisting of 30 μg/ml of activated DNA, 10 μg/ml of bovine serum albumin, 8 mM $MgCl_2$, 100 mM KCl, 50 μM of each dATP, dCTP and dGTP, and 1.3 μM [^{3}H]TTP (sp. act. 4000 cpm/pmole). Final volume was 250 μl. Other additions were as described under Results. The reaction was stopped at 10 min by pipetting 200-μl aliquots of the reaction mixture into 2 ml of ice-cold 12% trichloroacetic acid (TCA). The acid precipitate was collected on Whatman GF/C filters and was washed successively with 4 ml of 5% TCA (with 50 mM potassium pyrophosphate), 1 ml of ethanol, and 1 ml of ethyl ether. The filters were then dried, and the radioactivity was counted as described for mitochondrial protein synthesis. Correction for non-specific incorporation was made by subtracting from each assay the radioactivity found on a filter which received the same amount of protein and [^{3}H]TTP as the assays without incubation. Standard amounts of labeled TTP were counted under the same conditions and were used to compute pmoles of [^{3}H]TTP incorporated.

Protein determination. The amount of protein in the mitochondrial preparations was determined by the biuret method [8], using bovine serum albumin (Fraction V) as standard.

Materials. Ticlopidine and analogues (see Fig. 1) were provided by Clin-Midy/Sanofi, Montpellier, France. Radioactive compounds were purchased from the New England Nuclear Corp., Boston, MA. 2′,3′-Dideoxythymidine 5′-triphosphate was from P. L. Biochemicals, Inc., Milwaukee, WI. Other reagents were from the Sigma Chemical Co., St. Louis, MO, or were of analytical grade.

RESULTS

Effects of ticlopidine and analogues on oxidative phosphorylation. Using an NAD-linked substrate (glutamate) and an FAD-linked substrate (succinate) for mitochondrial oxidation, both State 4 and State 3 were recorded.

Traces A and C of Fig. 2 depict a representative experiment of well-coupled rat liver mitochondria when oxidizing the respiratory substrates glutamate and succinate. As shown, the addition of mitochondria resulted in a typical respiratory State 4 that was highly stimulated by the addition of ADP to generate State 3, during which ATP is synthesized. Also, as shown, 2,4-dinitrophenol caused uncoupling as expected. Ticlopidine (20 μg/ml) when added to

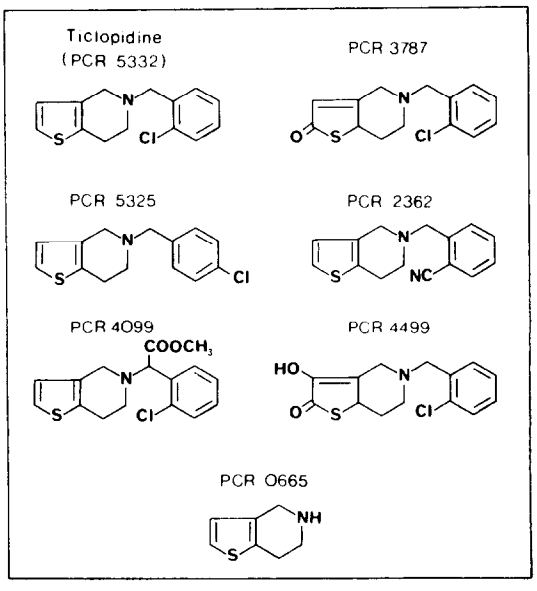

Fig. 1. Formulas of ticlopidine and some of its analogues used in the present study. PCR = Parcor.

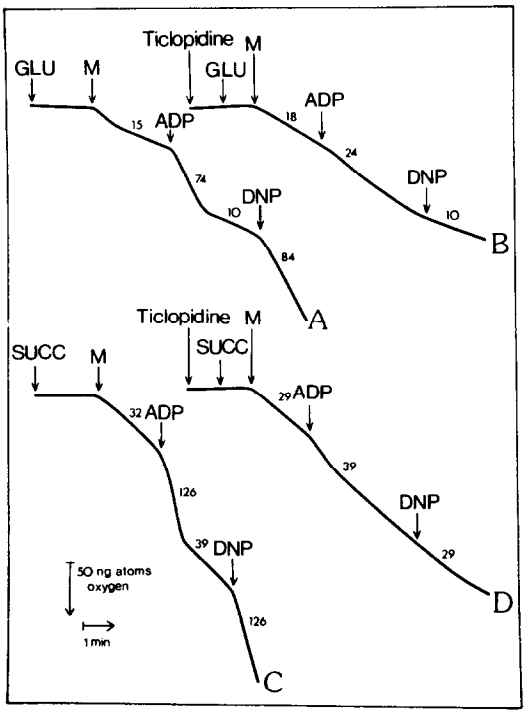

Fig. 2. Representative oxypolarographic tracings of rat liver mitochondria oxidizing glutamate or succinate in the absence and the presence of ticlopidine. Freshly isolated mitochondria (M) (1 mg protein) were incubated at 30° in 2 ml of a reaction medium consisting of 25 mM glycylglycine, 60 mM KCl, 87 mM sucrose and 5 mM phosphate (pH 7.4), with 10 mM of either glutamate (GLU) or succinate (SUCC). When present, 20 μg/ml of ticlopidine was added to the reaction medium as indicated. Other additions were 0.1 mM ADP and 0.1 mM 2,4-dinitrophenol (DNP). Traces A and C are controls. The numbers along the traces give the initial rates of oxygen consumption expressed in ng atoms oxygen·min^{-1}·(mg protein)$^{-1}$.

mitochondria oxidizing either glutamate or succinate (Fig. 2, Traces B and D respectively) had little or no effect on respiration but caused about 90% inhibition (see Methods and Materials for calculation) of both State 3 and the uncoupled state. These results are in agreement with previous work on ticlopidine [9].

Tested under similar conditions and at 20 μg/ml, the other analogues PCR 5325, PCR 4099, PCR 3787, PCR 2362, PCR 4499 and PCR 0665 showed little effect on State 4 (Table 1). At the higher concentration of 40 μg/ml, ticlopidine inhibited the respiration 38 and 45% with glutamate and succinate respectively. Other analogues, however, were much less inhibitory and some were actually slightly stimulatory (see Table 1).

The differential effects of ticlopidine and its analogues on mitochondrial respiratory State 3 are represented in Figs. 3 and 4, where the results are expressed as percent of control State 3 with glutamate and succinate, respectively, in the presence of various drug concentrations (ranging from 5 to 40 μg/ml). Comapred to its analogues, ticlopidine (PCR 5332) appeared to be the most toxic compound on mitochondrial ATP synthesis with either glutamate or succinate as energy source. PCR 0665 was

Table 1. Effects of 20 and 40 μg/ml of ticlopidine and its analogues on mitochondrial respiratory State 4 with glutamate and succinate*

Addition of PCR	Percent of control			
	Glutamate		Succinate	
	20†	40†	20	40
Ticlopidine (5332)	120 ± 1‡	62 ± 1	95 ± 5	55 ± 4
5325	100 ± 1	81 ± 1	116 ± 7	111 ± 1
4099	87 ± 2	100 ± 1	90 ± 2	79 ± 3
3787	87 ± 1	112 ± 1	105 ± 2	95 ± 2
2362	112 ± 1	112 ± 1	118 ± 2	113 ± 1
4499	100 ± 1	94 ± 1	121 ± 3	134 ± 3
0665	117 ± 6	118 ± 1	111 ± 4	108 ± 1

* Experimental conditions were as in Fig. 2.

† Twenty or forty μg drug/ml was present in the incubation medium before the addition of mitochondria.

‡ Values represent the mean of four to seven determinations ± S.E. Control specific activities for glutamate and succinate were 15 ± 1 and 33 ± 1 ng atoms oxygen·min^{-1}·(mg protein)$^{-1}$ respectively.

the least toxic, showing little or no effect. The other analogues caused different degrees of toxicity as shown in Figs. 3 and 4. For comparison, Table 2 gives the percent inhibition of State 3 produced by each drug when used at 20 μg/ml. At this concentration, the analogues fell in the order of their toxicity from the most to the least toxic as follows: PCR 5332 ≃ PCR 5325 > PCR 4099 ≥ PCR 3787 ≥ PCR 2362 > PCR 4499 > PCR 0665.

We also compared the effects of ticlopidine with those of chlorpromazine, an antipsychotic drug also occasionally associated with agranulocytosis. While chlorpromazine is known to affect certain mitochondrial functions [10, 11], the results shown in Fig. 5 (A and B) clearly demonstrate that ticlopidine and chlorpromazine (used at the same concentrations in μg/ml) differed in their action on mitochondria. Thus, while chlorpromazine (up to 40 μg/ml) produced a dose-related uncoupling-like action on respiration, ticlopidine at low concentration appeared to have slight or no effect on that activity, but at

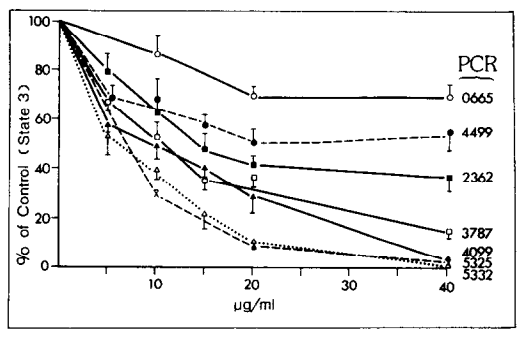

Fig. 3. Effects of different concentrations of ticlopidine and its analogues on mitochondrial respiratory State 3 with glutamate as substrate. Experimental conditions were as in Fig. 2. Values are means of three to seven assays ± S.E.

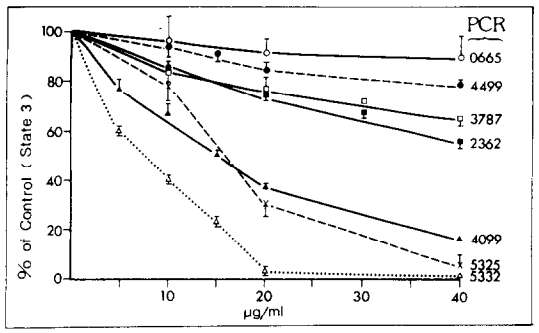

Fig. 4. Effects of different concentrations of ticlopidine and its analogues on mitochondrial respiratory State 3 with succinate as substrate. Conditions were as in Fig. 2.

higher concentration it was partially inhibitory (Fig. 5A). And at State 3, ticlopidine inhibition was much more pronounced than that of chlorpromazine (Fig. 5B).

Effects of ticlopidine and analogues on mitochondrial protein synthesis. The incorporation of [^{14}C]-L-leucine into rat liver mitochondrial protein was used to study mitochondrial protein synthesis. Incorporation was assayed in the presence of chloramphenicol, a known inhibitor of mitochondrial protein synthesis. Care was taken to run the assays under conditions similar to those used for the oxypolarographic study in terms of mitochondrial and drug concentrations per unit volume (see details under Methods and Materials). Table 3 shows that neither ticlopidine nor any of its analogues, used up to 40 μg/ml, exhibited any significant inhibitory effect on mitochondrial leucine incorporation. Rather, some of these drugs resulted in a slight enhancement of incorporation. In contrast, when assayed under the same experimental conditions, chloramphenicol at 10 μg/ml caused 80% inhibition of protein synthesis as expected.

Effects of ticlopidine and analogues on mitochondrial DNA polymerase activity. To assess whether ticlopidine may affect the mitochondrial DNA synthesis machinery, we tested its effect and

Table 2. Inhibition of mitochondrial oxidative phosphorylation by 20 μg/ml of ticlopidine and its analogues with glutamate or succinate as oxidative substrate*

Addition	Percent inhibition	
	Glutamate	Succinate
Ticlopidine (PCR 5332)	90	96
PCR 5325	90	75
PCR 4099	72	64
PCR 3787	64	23
PCR 2362	60	25
PCR 4499	50	16
PCR 0665	25	6

* The inhibitory effect was calculated from the data of Figs. 3 and 4. Control specific activities for glutamate and succinate were 70 ± 2 and 127 ± 3 ng atoms oxygen·min^{-1}·(mg protein)$^{-1}$ respectively.

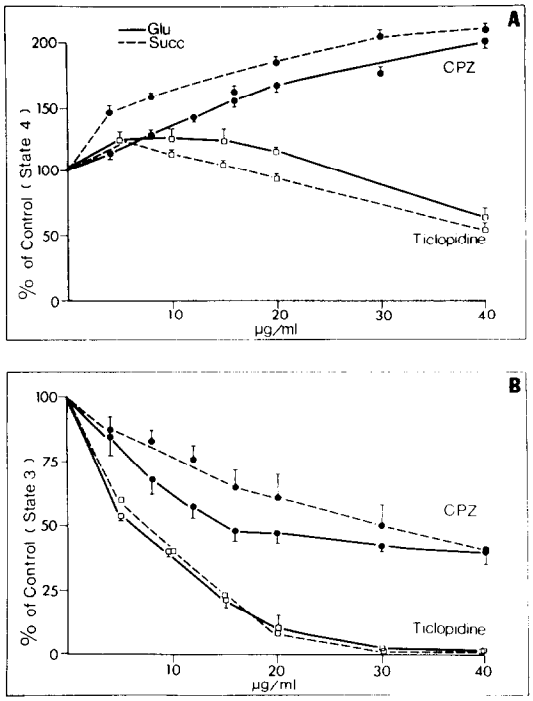

Fig. 5. Comparison between the effects of ticlopidine and chlorpromazine (CPZ) on mitochondria. (A) Effects of different concentrations on mitochondrial respiration (State 4) with glutamate or succinate. (B) Effects of different concentrations on oxidative phosphorylation (State 3) with glutamate or succinate. Conditions were as in Fig. 2. Values are means of three to seven assays ± S.E.

that of its analogues on the incorporation of [^{3}H]-TTP into activated calf thymus DNA as detailed in Methods and Materials. Ethidium bromide and dideoxythymidine triphosphate (ddTTP), known as specific inhibitors of mitochondrial DNA polymerase

Table 3. Effects of ticlopidine, its analogues and chloramphenicol on mitochondrial protein synthesis*

Addition	Concn (μg/ml)	cpm/filter disk	% Control
None		560 ± 30†	100
Ticlopidine	5	800 ± 30	143
(PCR 5332)	10	780 ± 30	139
	20	850 ± 30	152
	40	550 ± 30	98
PCR 5325	20	750 ± 30	134
	40	410 ± 40	73
PCR 4099	20	600 ± 60	107
	40	760 ± 40	136
PCR 3787	20	660 ± 30	118
	40	680 ± 20	121
PCR 2362	20	660 ± 40	118
	40	710 ± 50	127
PCR 4499	20	600 ± 40	107
	40	660 ± 60	118
PCR 0665	20	460 ± 30	82
	40	430 ± 30	77
Chloramphenicol	10	110 ± 10	20

* Experimental conditions were as described under Materials and Methods. Counting efficiency was 80%.
† Values are means of eight to fourteen assays ± S.E.

Table 4. Effects of ticlopidine, its analogues, ethidium bromide and ddTTP on mitochondrial DNA polymerase activity as determined by [^{3}H]TTP incorporation*

Addition	Concn (μg/ml)	% Control
None		100 ± 10†
Ticlopidine	20	99 ± 3
(PCR 5332)	40	100 ± 4
PCR 5325	20	91 ± 4
	40	101 ± 9
PCR 4099	20	99 ± 4
	40	119 ± 11
PCR 3787	20	123 ± 8
	40	127 ± 8
PCR 2362	20	109 ± 5
	40	127 ± 8
PCR 4499	20	129 ± 11
	40	122 ± 9
PCR 0665	20	120 ± 14
	40	112 ± 8
Ethidium bromide	9	17 ± 4
ddTTP‡	3.6	30 ± 11

* Experimental conditions were as described under Methods and Materials.

† Values are means of four to eight assays ± S.E. Control values varied between 2 and 4 pmoles [^{3}H]TTP incorporated/mg protein/10 min.

‡ ddTTP is 2′,3′-dideoxythymidine 5′-triphosphate.

activity [12–14], were used for comparison. As shown in Table 4, ticlopidine and analogues, used at concentrations up to 40 μg/ml, did not inhibit the TTP incorporation; rather some analogues slightly enhanced the activity. On the other hand, the addition of ethidium bromide and ddTTP at 9 and 3.6 μg/ml, respectively, produced the anticipated inhibitory effect.

DISCUSSION

The therapeutic use of the new drug ticlopidine has been extremely successful in controlling platelet aggregation [15]; however, some toxic side effects have been reported. One of the rare but serious complications of ticlopidine therapy is the injury of the hematopoietic tissue, resulting in granulocytopenia and pancytopenia [1, 2]. In view of the relationship of hematological toxicity to mitochondrial injury from some drugs such as chloramphenicol and gold compounds [16, 17], we have designed the present *in vitro* study to examine the interaction of ticlopidine and some of its analogues (cf. Fig. 1) with key mitochondrial functions.

When oxidative phosphorylation, one of the most important mitochondrial functions, was activated by the addition of either an NAD (glutamate)- or an FAD (succinate)-linked substrate, ticlopidine and analogues showed no inhibitory effect on respiratory State 4. At the relatively high concentration of 40 μg/ml, only ticlopidine showed 38–45% inhibition of that state and, while PCR 5325 is structurally similar to ticlopidine, the different effect of these two drugs when tested at 40 μg/ml on State 4 with succinate (Table 1) is not readily explained. In contrast, the respiratory State 3, during which mitochondria synthesize ATP, was inhibited in a concentration-dependent manner with 90% inhibition at 20 μg/ml of the drug. With State 3, the analogue PCR 5325 was as effective as ticlopidine. Other analogues were less effective. Thus, the inhibition by 20 μg drug/ml ranged from 72% with PCR 4099 to 6% with PCR 0665. This decreasing order of inhibition was more pronounced with succinate than with glutamate, where the same concentration of the drug appeared always less inhibitory.

Assayed under similar experimental conditions, chlorpromazine, which also causes granulocytopenia, affects mitochondria differently from ticlopidine. Whereas chlorpromazine caused concentration-dependent activation of respiratory State 4, ticlopidine in a higher concentration range caused inhibition. In addition, ticlopidine was twice as inhibitory on State 3 as chlorporomazine.

The fact that mitochondrial protein synthesis was not inhibited by ticlopidine and its analogues, even at the high concentration of 40 μg/ml, suggests that these drugs do not have the same site of interaction with the mitochondria as chloramphenicol. The slight activation of leucine incorporation observed with ticlopidine (at up to 20 μg/ml) and some other analogues (Table 3) might have been due to a drug-related change in the mitochondrial membrane permeability, which could facilitate the transport of one or more substances related to protein synthesis.

Mitochondrial DNA polymerase activity was essentially unaffected by ticlopidine and its analogues. It should be noted that the polymerase activity was assayed without interference of mitochondrial membranes which were removed by ultracentrifugation of sonicated mitochondria before assay. Under these conditions, even high concentrations of ticlopidine or its analogues did not cause any inhibition of thymidine triphosphate incorporation into DNA.

The relationship of the *in vitro* inhibition of oxidative phosphorylation by ticlopidine observed herein to *in vivo* toxicity from the drug cannot be ascertained at present. Plasma drug concentrations ranges between 2 and 7 μg/ml depending on route of administration [15]. Although little inhibition was observed at these concentrations *in vitro*, the possibility of intracellular drug accumulation *in vivo* either by an active process and/or by compartmentalization cannot be excluded.

In conclusion, of the major mitochondrial functions studied, only oxidative phosphorylation, which is a membranous function, was inhibited by ticlopidine and some of its analogues. Ticlopidine and PCR 5325 were the most toxic, while PCR 0665 (which lacks the —CH_3- and —Cl-substituted benzene ring of ticlopidine) was the least toxic to that function. The exact mechanism of inhibition remains to be determined. The drug may (a) interfere with one or more reaction(s) of the energy conserving mechanism, (b) affect the permeability of the mitochondrial inner membrane, or (c) increase the fluidity of that membrane as suggested for erythrocytes [18]. These possibilities and others are currently under investigation.

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