# Nonreceptor Interactions in the Pharmacology of Blood Platelets

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**Abstract.** On the basis of values corresponding to concentrations exhibiting 50% inhibition of platelet aggregation induced with different stimuli and 50% inhibition of arachidonic acid liberation and thromboxane generation, we compared the antiplatelet effect of two cationic amphiphilic drugs – chloroquine and dithiaden Compared to chloroquine, dithiaden was much more effective in inhibiting platelet aggregation, A-23187 induced arachidonic acid liberation and thromboxane generation Chloroquine, on the other hand, was more effective in the inhibition of thrombin-induced arachidonic acid liberation

**Key words:** Human platelets — Aggregation — Phospholipase $A_2$ — Thromboxane — $ED_{50}$ values

## Introduction

Blood platelets respond to different stimuli by adhering to the foreign surface, aggregating and liberating biologically active substances Platelet aggregation *in vivo* and *in vitro* is decreased or abolished with many pharmacologically active substances Of these cationic amphiphillic drugs (CAD) are of utmost importance (Lasslo 1984, Schror 1995) CAD from different pharmacological groups were demonstrated to decrease dose-dependently the activation of platelet aggregation induced with different stimuli (Jančinová et al 1994, Nosáľ 1995, Nosáľ et al 1992, 1997) In this study we investigated and compared the $ED_{50}$ values for inhibition of aggregation, arachidonic acid liberation (phospholipase $A_2$-$PLA_2$ activity) and thromboxane generation of the histamine $H_1$-receptor antagonist dithiaden (DIT) and the antimalarial – antiinflammatory chloroquine (CQ) in human blood platelets

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## Materials and Methods

*Blood sampling, platelet isolation and aggregation*

Blood was taken at the blood bank from healthy volunteers (men, aged 20 to 50 years) by antecubital venepuncture and was immediately mixed with 3 8% v/w trisodium citrate(dihydrate) in the ratio of 9 ml blood to 1 ml citrate Blood was centrifuged in polypropylene tubes for 15 min at 200 × $g$ and 22 °C Platelet rich plasma (PRP) and isolated platelets were prepared by differential centrifugation and their number for aggregation was adjusted respectively with platelet poor plasma or Tyrode solution to get 200,000 platelets/$\mu$l of sample (for details see Nosáľ et al 1997) The aggregation procedure was as follows 450 $\mu$l platelet suspension, stabilisation in an aggregometer (Aggrometer Chrono-log, USA) at 37 °C for 1 min, addition of the drug tested (20 $\mu$l), incubation for 30 s, registration of aggregation curves up to 240 s following stimulus administration Inhibition of platelet aggregation was calculated from the aggregation curves as the amplitude (mm) in the 30th s and evaluated by means of a dose-response curve (Jančinová et al 1994)

*Phospholipase $A_2$ activity ($^3$H-arachidonic acid liberation)*

The PRP was mixed with 4 5% v/w citric acid and 6 6% v/w dextrose in the amount of 50 $\mu$l per 1 ml PRP and with [$^3$H]-arachidonic acid (AA) at 1 85 × $10^4$ Bq/ml of PRP After 60 min of incubation at 37 °C, the samples were centrifuged for 10 min at 980 × $g$ at 22 °C and platelets were washed two times by centrifugation in an equal volume of Tyrode buffer with EDTA for 6 min at 980 × $g$ at 22 °C Samples were treated and the radioactivity was measured by means of liquid scintillation counting (Packard Tricarb 2500 scintillation counter) as described previously (Nosáľ et al 1997)

*Thromboxane ($TXB_2$) generation*

Platelets were isolated and prepared by the same procedure as described for [$^3$H]-AA liberation Platelets were diluted with EDTA-free Tyrode buffer to get 10,000 platelets/$\mu$l of the sample Tubes with 450 $\mu$l of platelet suspension were stabilised at 37 °C for 2 min and treated subsequently with 20 $\mu$l of the drug tested for 30 s Stimulation with either 20 $\mu$l of A23187 (1 8 $\mu$mol/l) or with 20 $\mu$l of thrombin (0 05 NIH U/ml) followed for 5 min at 37 °C Incubation was stopped by addition of indomethacine (0 1 mmol/l – final concentration), cooling and centrifugation of the tubes Thromboxane was determined in the supernatant by means of $TXB_2$-RIA kit as described earlier (Nosáľ et al 1992)

*Evaluation of $ED_{50}$ data*

For inhibition of aggregation (5 to 7 measurements), arachidonic acid liberation (5 to 6 measurements) and thromboxane generation (4 to 5 measurements) of platelets pretreated with DIT or CQ and subsequently stimulated with different stimuli (see results), the mean effective concentrations ($ED_{50}$ ) were calculated from the mean dose-response curves by means of a PC software program "Statistica" The significance of $ED_{50}$ data was established by using Student's $t$-test

*Materials*

Chloroquine® ACO Sweden, Dithiaden® Lečiva Praha, Czech Republic, ADP, SDS Serva, Germany, epinephrine bitartrate (adrenaline) Sigma, USA, calcium ionophore A23187 Calbiochem, Switzerland, human thrombin Imuna Š Michaľany, Slovakia, 5,6,8, 9,11,14,15-N-[$^3$H] arachidonic acid (7 TBq/mmol) and [$^{125}$I] $TXB_2$ RIA kit a kind gift from Dr I Mucha, Institute of Radioizotopes, Budapest, Hungary

All other chemicals of analytical grade were from available commercial sources.

## Results

The $ED_{50}$ values for CQ and DIT to inhibit platelet aggregation stimulated with ADP, ADR, THR and Ca-ionophore A23187 are demonstrated in figure 1. It is evident from the figure that DIT compared to CQ is much more potent in inhibiting platelets stimulated for aggregation with different stimuli. With $ED_{50}$ values given in brackets, DIT ($\mu$mol/l) inhibited aggregation in the following rank order of stimuli: A23187 (14.7 ± 1.2) < thrombin (44.7 ± 2.0) < adrenaline (47.3 ± 2.0) < ADP (87.2 ± 2.2). The rank order of potency for CQ $ED_{50}$ ($\mu$mol/l) to inhibit platelet aggregation was: thrombin (99.5 ± 2.5) > adrenaline (100.5 ± 2.3) > ADP (120 ± 3.5) > A23187 (165.0 ± 3.8).

Figure 2 shows the $ED_{50}$ values for the inhibition of arachidonic acid liberation and thromboxane generation in platelets pretreated with CQ and DIT, subsequently stimulated with thrombin and A23187. The $ED_{50}$ values for arachidonic acid liberation and $TXB_2$ generation in CQ pretreated and thrombin stimulated platelets were 6.3 ± 1.0 and 8.5 ± 0.7 $\mu$mol/l, respectively. On the other hand, the respective $ED_{50}$ values for AA liberation and $TXB_2$ generation in CQ pretreated and A23187-stimulated platelets were 191.2 ± 27.4 and 138.8 ± 3.7 $\mu$mol/l.

In DIT-pretreated and thrombin-stimulated platelets the $ED_{50}$ values for AA liberation and $TXB_2$ generation were 64.2 ± 2.3 and 1.5 ± 0.2 $\mu$mol/l, respectively. The respective $ED_{50}$ values in DIT pretreated and A23187-stimulated platelets for AA and $TXB_2$ generation were 46.1 ± 1.6 and 17.3 ± 1.5 $\mu$mol/l.

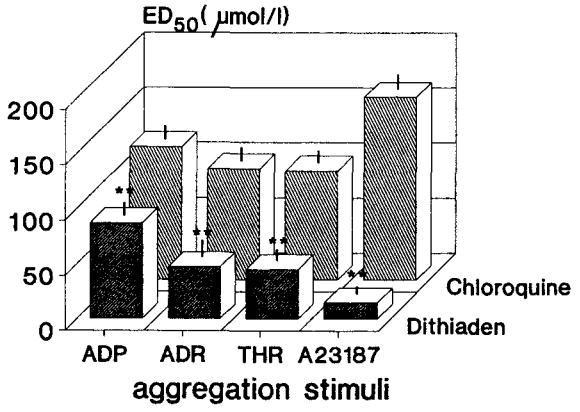


**Figure 1.** Antiaggregatory effect of Dithiaden and Chloroquine on human blood platelets stimulated with adenosine diphosphate (ADP), adrenaline (ADR), thrombin (THR) and $Ca^{2+}$-ionophore A23187 as evaluated by means of $ED_{50}$ values ($\mu$mol/l). $n = 5$ to 7 ± S.E.M. $^{**}p < 0.01$

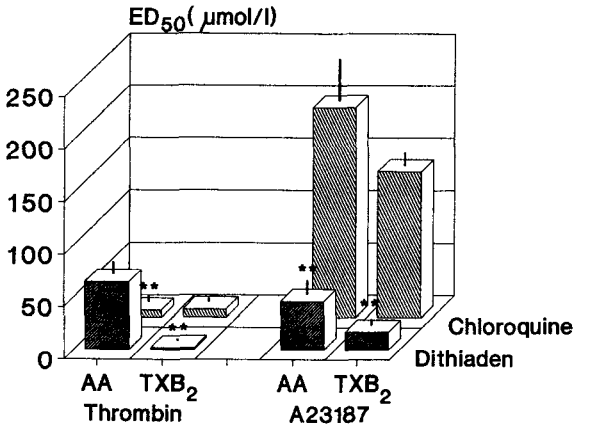


**Figure 2.** Effect of Dithiaden and Chloroquine on thrombin- and $Ca^{2+}$-ionophore A23187-induced phospholipase $A_2$ activity (measured as $^3$H-arachidonic acid liberation – AA) and thromboxane generation (measured as $TXB_2$). Results represent $ED_{50}$ values in µmol/l, $n = 4$ to 5 ± S.E.M. $^{**}p < 0.01$

## Discussion

In the light of $ED_{50}$ values, DIT and CQ differ in their potency to abolish platelet aggregation.The applied stimuli ADP, adrenaline, thrombin and $Ca^{2+}$-ionophore A23187 differ in their mechanism of platelet activation (Siess 1991). It is evident from the $ED_{50}$ data that DIT was much more effective than CQ in inhibiting secondary platelet aggregation. CQ, an antimalarial drug with antiinflammatory properties, and the $H_1$-histamine antagonist DIT decreased dose-dependently human platelet aggregation and inhibited the arachidonic acid pathway in platelets stimulated with thrombin and A23187 (Jančinová et al. 1997; Nosáľ et al. 1997).

The multipotent antiplatelet effect of CQ and DIT against different stimuli indicated that both drugs would act rather at signal transduction pathways than on particular receptors. Moreover, human platelets bear preferentially $H_2$- to $H_1$-receptors and no specific receptors for CQ have been demonstrated as yet (Launay et al. 1994; Nosáľ et al. 1995). In platelets, the signal transduction for second messengers is rather complex, with intraplatelet $Ca^{2+}$-mobilisation and $PLA_2$ activation playing the pivotal role (Rink and Sage 1990; Siess 1991; Spiegel 1987). Stimuli used in our experiments are known to raise cytosolic $Ca^{2+}$, important for $PLA_2$ activation (Kramer et al. 1989).

Both DIT and CQ dose-dependently inhibited thrombin- and A23187-stimulated activation of $PLA_2$ and TX generation (Nosáľ et al. 1995; 1997). As evident from $ED_{50}$ values, DIT was more potent in inhibiting $TXB_2$ generation, compared with inhibition of arachidonic acid liberation in thrombin- and A23187-stimulated platelets. This indicated that besides decreasing $PLA_2$ activity, DIT might also

interfere with platelet aggregation by inhibiting thromboxane synthase, as demonstrated for other CAD drugs (Mehta and Nichols 1990). In thrombin-stimulated platelets, CQ effectively decreased $PLA_2$ activity and TX generation in concentrations corresponding to $ED_{50}$ values of 6.3 and 8.3 $\mu$mol/l, respectively. These findings confirmed the suggested inhibitory mechanism of CQ on $PLA_2$ in other tissues (El Tahir 1987; Kubo and Hostetler 1985) and our hypothesis about CQ-induced inhibition of platelet aggregation (Nosáľ 1995, Nosáľ et al. 1995).

In A23187-stimulated platelets, 16 to 30 times higher concentrations were needed to produce $ED_{50}$ values for inhibition of TX generation and $PLA_2$ activation. It has been suggested that A23187 bypassing membrane receptors increases active cytoplasmic $Ca^{2+}$, which in turn activates cytosolic $PLA_2$ with subsequent increase in arachidonic acid cleavage from membrane phospholipids and eventual rise in thromboxane generation (Kramer et al. 1989, Kroll and Schafer 1989). Yet on human platelets a relative independence of $PLA_2$ of $[Ca^{2+}]_i$ was demonstrated (Zovoico et al. 1986), which may in part explain the lower effectiveness of CQ on A23187-induced platelet aggregation and arachidonic acid pathway mobilisation.

Platelet aggregation and secretion were shown to be rather complex events, yet neither is their inhibition a simple process. This was also demonstrated in this study by $ED_{50}$ values for inhibitory effect of two different drugs of cationic amphiphilic structure. Both drugs were effective: first, against stimuli with distinguished mode of action, second, at different concentrations, and third, at different sites of arachidonic acid pathway. These findings might contribute to the understanding of mechanisms of side and adverse effects of drugs from different pharmacological groups.

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