SYNTHESIS AND THROMBOLYTIC ACTIVITY
OF NEW THIENOPYRIMIDINONE DERIVATIVES

It has been observed that ticlopidine and clopidogrel show, apart from their delayed antiplatelet properties, an immediate and transient thrombolytic action related to the ability of these thienopyridines to stimulate the secretory function of vascular endothelium. With the objective to construct new molecules with identical thrombolytic potency but at a higher level, we carried out different structural modifications in the thienopyridine chemical molecule to conclude that the presence of a second N atom in the pyridine cycle (yielding pyrimidine moiety) and the presence of an additional cycle fused to the thienyl ring would lead to enhanced thrombolytic effects. Here we report the six-step synthesis of a series of new benzothienopyrimidinone derivatives characterized by this searched for potent thrombolytic activity. The pharmacological assay used anaesthetised Wistar rats with extracorporeal circulation in which arterial blood superfused thrombi adhering to a strip of collagen. Weight of thrombi was continuously monitored. Six compounds of the series were much more potent thrombolytic agents than their thienopyridine references: the effective thrombolytic dose that produced 30\% of maximum thrombolysis (ED$_{30}$) was at a range of 8 to 170 $\mu$g kg$^{-1}$ as compared with ED$_{30}$ values of 16000 to 20000 $\mu$g kg$^{-1}$ for clopidogrel and ticlopidine respectively. Especially with the most active compound, this difference in the threshold thrombolytic dose, giving an intensity of action higher by three orders of magnitude, was accompanied by a lengthening of the response. Apart from that these compounds have shown to be synthetic thrombolytics, they certainly deserve further studying.

Key words: antiplatelet thienopyridines; ticlopidine; clopidogrel; thienopyrimidinone derivatives synthesis; synthetic thrombolytics; vascular endothelium.
INTRODUCTION

During the last three decades of the 20th century, an intense research activity in the antithrombotic field was devoted to compounds showing antiaggregatory potency. Several drugs were explored, but except aspirin, among the huge number of synthetic molecules tested, only very few of them found a clinical use. Ticlopidine i.e. [5-(2-chlorophenyl)-methyl]-4,5,6,7-tetrahydro-thieno[3,2-c] pyridine hydrochloride (1) was first used in 1978 and finds a broad scope of applications (2,3). The mechanism of its antiaggregant activity depends on its antagonism to ADP membrane platelet receptors (4). This antiplatelet effect cannot be observed in vitro and is detectable ex vivo only many hours after oral administration, probably acting through an unstable metabolite (3).

However, apart from its delayed activity, it was first observed in 1996 (5) that ingestion of ticlopidine, at a dose of 500 mg by patients with peripheral arterial disease, was accompanied by an immediate fibrinolytic action, shown by shortening of euglobulin clot lysis time. The authors completed these results with the observation that in cats with extracorporal circulation (5,6), ticlopidine would cause antithrombotic and instantaneous transient thrombolytic effect on a preformed thrombus.

Another synthetic molecule, clopidogrel i.e. thieno [3,2-c] pyridine-5(4H)-acetic acid, α(2 chlorophenyl)-6,7-dihydro-,methyl ester,(S)- (7) is available since 1998 as an antiplatelet drug used in prevention or treatment of myocardial infarction and other diseases associated with atherosclerosis (8-11).

Both clopidogrel and ticlopidine share their chemical formulae with thieno- and hydrogenated-pyrido fused heterocycles (Fig 1) so they are usually called antiplatelet thienopyridines; their beneficial actions are linked with their ability to antagonise platelet ADP receptors which occur exclusively in vivo after their biotransformation to active and unstable metabolites (12). The difference between ticlopidine and clopidogrel chemical structure consists in the replacement of a CH₂ group by a CH-COOCH₃ group of atoms, so the molecule exists in two enantiomeric forms; the (S) enantiomer is the active compound (clopidogrel) whereas the (R) enantiomer is deprived of antiplatelet potency (13).

These new data on thienopyridines arose an interest to know whether the two enantiomers exert the thrombolytic effect previously shown by ticlopidine. Indeed, both enantiomers produced equal and immediate thrombolysis ex vivo in rats experiments in extracorporal circulation (14,15). The thrombolytic activity of (R) enantiomer deprived of antiplatelet effect demonstrates that ex vivo dispersion of thrombi by thienopyridines is not associated with their antiplatelet properties. The additional question was to know whether the thrombolytic effect of these thienopyridinic molecules was to be found in other chemically related compounds and whether a possible enhancement of thrombolytic activity could be linked with changes in their chemical structure. In a previous chemical-pharmacological study (14) we observed that a series of compounds where pyridine ring was
replaced by pyrimidine or pyrimidinone moieties left active derivatives where thrombolytic potencies were at least at the level of that for thienopyridines but for four of them became 10 to 30 times higher than that of ticlopidine. These data brought a qualitative response about the possibility to find thrombolytic potency in new synthetic molecules and justify further research on their activity.

This was the aim of the present study where we describe the synthesis (Fig. 2) of a series of new compounds (Table 1) and we report the pharmacological evaluation (Fig. 3) of their thrombolytic activity (Table 2), (Fig. 4).

Fig. 1 Chemical structures of antiplatelet thienopyridines and thienopyrimidinone derivatives.

Fig. 2 Synthesis of 3-substituted benzo[b]thieno[2,3-d]pyrimidin-4(3H)one (compounds C) and 3-substituted 1,2-dihydrobenzo[b]thieno[2,3-d]pyrimidin-4(3H)one (compounds D).
Table 1 – Yield, melting point (M.P.) and spectroscopic characterisation values of new thieno [2,3-d] pyrimidin-4(3H)-one derivatives.

<table>
<thead>
<tr>
<th>R</th>
<th>Yield %</th>
<th>M.P. °C</th>
<th>IR&lt;sub&gt;νmax&lt;/sub&gt; cm&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>&lt;sup&gt;1&lt;/sup&gt;H MNR δ (ppm)</th>
<th>Yield %</th>
<th>M.P. °C</th>
<th>IR&lt;sub&gt;νmax&lt;/sub&gt; cm&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>&lt;sup&gt;1&lt;/sup&gt;H MNR δ (ppm)</th>
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<tr>
<td>H</td>
<td>76</td>
<td>152</td>
<td>1660</td>
<td>8.23, 5.26, 7.5-8.7</td>
<td>85</td>
<td>155</td>
<td>1605</td>
<td>4.71, 4.63, 7.1-8.1</td>
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<tr>
<td>Cl(2)</td>
<td>41</td>
<td>131</td>
<td>1660</td>
<td>8.43, 5.46, 7.5-8.8</td>
<td>71</td>
<td>191</td>
<td>1610</td>
<td>4.80, 4.70, 7.4-8.2</td>
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<tr>
<td>CH&lt;sub&gt;3&lt;/sub&gt;(3)</td>
<td>68</td>
<td>160</td>
<td>1660</td>
<td>8.23, 5.20, 7.1-8.6</td>
<td>72</td>
<td>199</td>
<td>1600</td>
<td>4.70, 4.60, 7.2-8.2</td>
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<tr>
<td>F(2)</td>
<td>65</td>
<td>153</td>
<td>1670</td>
<td>8.20, 5.20, 7.3-8.6</td>
<td>61</td>
<td>201</td>
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<td>4.76, 4.66, 7.3-8.2</td>
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<td>61</td>
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<td>1675</td>
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<td>78</td>
<td>167</td>
<td>1610</td>
<td>4.86, 4.66, 6.9-7.4</td>
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Fig.3 Original tracings of thrombolytic (THR) responses and blood pressure (BP) responses in four rats which received intravenous injections of a dose of 30000 μg/kg i.v. of ticlopidine or clopidogrel or a dose of 30 μg/kg i.v. of one of the two most potent thienopyrimidinone derivatives (compounds D<sub>I</sub> and D<sub>II</sub>, see Table 2)
Table 2 – Chemical structure and thrombolytic activity of new thienopyrimidinone derivatives and antiplatelet thienopyridines references thrombolytic activity.

THIENOPYRIMIDINONE DERIVATIVES

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>R</th>
<th>Name</th>
<th>ED$_{50}$ µg kg$^{-1}$ i.v.</th>
<th>Number of doses tested</th>
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<td>D$_I$</td>
<td>H</td>
<td>3-(phenyl)-methyl-1,2-dihydrobenzo[b]thieno[2,3-d]pyrimidinone-4(3H) one</td>
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<td>D$_{II}$</td>
<td>Cl(2)</td>
<td>3-[(2-chlorophenyl)-methyl] 1,2-dihydrobenzo[b]thieno[2,3-d]pyrimidinone-4(3H) one</td>
<td>30.0</td>
<td>28</td>
</tr>
<tr>
<td>D$_{III}$</td>
<td>CH$_3$(3)</td>
<td>3-[(3-methylphenyl)-methyl] 1,2-dihydrobenzo[b]thieno[2,3-d]pyrimidinone-4(3H) one</td>
<td>140.5</td>
<td>24</td>
</tr>
<tr>
<td>D$_{IV}$</td>
<td>F(2)</td>
<td>3-[(2-fluorophenyl)-methyl] 1,2-dihydrobenzo[b]thieno[2,3-d]pyrimidinone-4(3H) one</td>
<td>55.9</td>
<td>23</td>
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<tr>
<td>D$_V$</td>
<td>CH$_3$O(2)</td>
<td>3-[(2-methoxyphenyl)-methyl] 1,2-dihydrobenzo[b]thieno[2,3-d]pyrimidinone-4(3H) one</td>
<td>39.9</td>
<td>23</td>
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<tr>
<td>D$_{VI}$</td>
<td>CF$_3$(2)</td>
<td>3-[(2-trifluoromethylphenyl)-methyl] 1,2-dihydrobenzo[b]thieno[2,3-d]pyrimidinone-4(3H) one</td>
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THIENOPYRIDINE REFERENCES

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<tr>
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<tr>
<td>Ticlopidine</td>
<td>20194</td>
<td>24</td>
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<tr>
<td>Clopidogrel</td>
<td>15955</td>
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MATERIALS AND METHODS

Drugs and reagents

Ticlopidine and clopidogrel were kindly donated by Sanofi Recherche (Toulouse, France). New thienopyrimidinone derivatives were synthesised in the Laboratory of pharmaceutical organic chemistry at Bordeaux II University (Bordeaux, France).

Chemical synthesis

Starting material was ethyl 2-amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (compound A) which was aromatised by action of sulphur heated during 8 hours at 220°C in
dimethyl phtalate solution according to a three step reaction (16,17) because it is necessary to protect the amino group by prior acylation and to regenerate it after aromatisation. Then, using ethyl 2-aminobenzo[b]thiophene-3-carboxylate (compound B) obtained, cyclisation of pyrimidinone ring was achieved by simultaneous action (18) of both appropriate substituted benzylamine and triethylorthoformiate heated for 15 hours in decaline at 160°C. The 3-substituted benzo[b]thiophene[2,3-d]pyrimidin-4(3H)-ones obtained (compounds C) were then 1,2-dihydrogenated by action of lithium aluminium hydride in tetrahydrofurane at 60°C during 2 hours (19) to give 3-substituted 1,2-dihydrobenzo[b]thiophene[2,3-d]pyrimidin-4(3H)-ones (compounds D), (Fig.2).

Melting points were determined on a Kofler apparatus. IR spectra were recorded on a Shimadzu IR 470 spectrometer as potassium bromide pellets and frequencies (ν) are expressed in cm⁻¹. ¹H-NMR spectra were obtained on a Varian EM 360 L spectrometer in CDCl₃ with TMS as the internal standard and chemical shifts are reported in ppm (δ).

Other chemicals were purchased from Sigma Aldrich France.

**Bringing compounds into solution**

Thienopyridines were dissolved with vigorous stirring in saline at 37°C, while synthesised compounds were dissolved *ex tempore* in dimethyl sulphoxide (DMSO). The investigated compounds were injected intravenously at doses ranging from 3 μg kg⁻¹ to 30 mg kg⁻¹. Any dose of a synthesised compound was given in a volume of 15 μL DMSO. DMSO alone injected intravenously at a volume of 20 μL did not produce in rats detectable effect on blood pressure (BP) or weight of thrombus (THR).
**Thrombolysis in extracorporal arterial circulation in Wistar rat**

Thrombolytic effect (i.e. dispersing preformed blood clots in flowing arterial blood) exerted by drugs which are administrated intravenously to experimental animals was assayed *ex vivo* according to the methods described for cats (6) and adapted to rats (5,20). Wistar rats weighting 400-500 g, anaesthetised with thiopental (800 U kg⁻¹, intravenously) were used. For monitoring mean arterial blood pressure (BP), the Isotec type electric transducer was connected by a cannula to the left carotid artery, whereas the cannulated right carotid artery was delivering blood into extracorporal circulation by a peristaltic pump (37°C, 1 ml min⁻¹). Blood after superfusing a collagen strip (cut from rabbit’s Achilles tendon) was returned to circulation through the cannulated left femoral vein. During superfusion, a clot was formed on the surface of collagen strip and its weight was continuously monitored using an auxotonic 386 Harvard transducer. After 20-30 minutes of superfusion, the strip gained in weight by about 100 mg and stayed at that level during next 3-5 hours of experiment. The loss in weight of thrombus after intravenous drug injection characterised its thrombolytic response. The structure of these thrombi was studied using both light and electron microscopy (6,21) and showed presence of platelets aggregates, scanty fibrin patches and a number of leucocytes and erythrocytes.

The first pilot approach consisted of finding minimal thrombolytic doses of tested compounds which were injected at a range of doses from 1 μg kg⁻¹ i.v. to 30 μg kg⁻¹ i.v. to 2-3 rats. When the minimal effective dose was established then that minimal dose [Min D] and 3 x [Min D] and 10 x [Min D] were injected intravenously to at least seven rats, however, each dose was given to a single rat only. The effects on BP and TRH were recorded. The thrombolytic effect of thienopyrimidinone derivatives was read one hour after injection of the compound. Each dose was tested separately in the group of 7-11 rats and these data were used to calculate the effective thrombolytic dose (μg kg⁻¹) of each compound that produced 30 % of maximal thrombolysis (ED₃₀) obtained from the equation of regression lines (Table 2), (Fig. 4). In some experiments, the observation was extended up to three hours after injection and the duration of thrombotic response of thienopyrimidinone derivatives was recorded (Fig.3). Our reference drugs i.e. ticlopidine and clopidogrel were treated in a similar way. The difference was that their threshold thrombolytic dose appeared to be higher by three orders of magnitude (Table 2).

**Statistical analysis**

Arithmetic means are given with their standard errors (M ± SEM). Differences inside group were assessed by paired Student’s t test, while for statistical analysis between groups unpaired t test was used sometimes, however, we mostly applied one-way analysis of variance (ANOVA) followed with Scheffe’s *a posteriori* test (for n groups > 2). P values were calculated.

All procedures were carried out according to EU directives and reviewed by local ethical committee.

**RESULTS**

Reference compounds, i.e. ticlopidine and clopidogrel produced thrombolysis at ED₃₀ of 20194 μg kg⁻¹ i.v. and 15955 μg kg⁻¹ i.v. respectively, (Table 2) These thrombolytic effects lasted 1h - 1.5h (Fig.3). All of new D compounds were much more potent thrombolytic agents as compared to the reference compounds. The most potent thrombolytic agent was compound Dᵥ₁ with ED₃₀ = 8 μg kg⁻¹ i.v. (i.e.
2000 times more potent thrombolytic agent than clopidogrel). At doses 3 - 30 μg kg⁻¹ i.v. compound D VI hardly influenced BP and at that range of doses, the persistence of thrombolysis by compound D VI was longer than three hours of the observation period. As to the potency of thrombolytic action, next was compound D II with ED₃₀ = 30 μg kg⁻¹ i.v., however, the essential difference between compounds D II and D VI was duration of their thrombolytic actions. The thrombolytic action of compound D II at all three doses tested, i.e. 10, 30 and 100 μg kg⁻¹ i.v., lasted only 1.5 - 2.5 hours after its administration. Compound D V was the closest to compound D II, with ED₃₀ = 33.9 μg kg⁻¹ i.v. and duration of thrombolytic action of 2 hours after a 100 μg kg⁻¹ i.v. Marginally less potent than compound D V were compounds D IV, D III, and D I with their ED₃₀ = 55.9 μg kg⁻¹ i.v., 140.5 μg kg⁻¹ i.v., and 170 μg kg⁻¹ i.v. respectively. Also, the duration of their thrombolytic action was closer to that of compound D II rather than that long lasting effect of compound D VI. Indeed compounds D IV, D III and D I showed thrombolysis of duration 2 - 2.5 hours.

DISCUSSION

Classic thienopyridines (ticlopidine and clopidogrel) are highly successful antiplatelet drugs for treatment of patients with atherothrombosis. Their beneficial clinical properties are usually attributed to blockade of platelet P₂Y₁₂ receptors for ADP by their unstable metabolites. Activation of these receptors by ADP is associated with the Gi protein-mediated inhibition of adenylate cyclase, fall in cyclic-AMP, followed by mobilisation of [Ca²⁺], exposure of glycoprotein IIb/IIIa receptors and formation of stable platelet aggregates (12,23). In patients and animals, the above effects of thienopyridines appear after a considerable delay required for hepatic metabolism of their native molecules to active unstable antiplatelet metabolites. Previously we reported on immediate thrombolytic effects of thienopyridines and we claimed that this action was related to the release of endothelial prostacyclin (PGI₂), nitric oxyde (NO) or plasminogen activator (t-PA) (5,14,15). Also in vitro, cultured endothelial cells when stimulated by thienopyridines released 6-keto-PGF₁₀ and nitrite – the degradation products of PGI₂ and NO (22). At that stage it appears that ticlopidine and clopidogrel show two pharmacological properties which are independent from each other, i.e. the antiplatelet effect mediated by their unstable metabolites, and the thrombolytic action depending on activation of the secretory function of vascular endothelium by the native chemical moieties (14,20). The participation of both pharmacological mechanisms of thienopyridines in their clinical efficacy remains an unanswered question (5), although clopidogrel and its enantiomer (being inactive as antiplatelet agent) both are equally potent thrombolytics in vivo (14).

In an attempt to address the above question we synthesised thienopyridine-like compounds with stronger thrombolytic potencies than those for ticlopidine and
clopidogrel (14). The chemical changes were as follows: a) pyridine ring was replaced by pyrimidine ring and b) thienyl ring was condensed with various cyclic structures. Presently, in addition to a thienyl ring condensed with a benzene ring, the \( N_3 \)-attached benzyl moiety was substituted in the ring with various chemical groups. The compounds obtained in this way were assayed for their thrombolytic potencies in our rat model in vivo (20). Their \( ED_{50} \) values for thrombolysis ranged from 8 to 170 \( \mu g \ kg^{-1} \) i.v. as compared to 16 or 20 mg kg\(^{-1}\) i.v. for clopidogrel or ticlopidine, respectively. In some cases (compound D\( \alpha \)) also the duration of thrombolysis exceeded that for classical thienopyridines. The mechanism of the ex vivo thrombolytic activity of these new compounds remains unknown but as it takes place immediately after their intravenous administration, we suppose that thrombolysis is associated with their native molecules. It is reasonable to assume that endothelial mechanisms (PGI\(_2\) release) are involved similarly as in the case of thienopyridines (22), however, an additonal 6-keto-PGF\(_{1\alpha}\) assay is required. The powerful thrombolytic action of these new \( N_3 \)-benzyl substituted benzothienopyrimidinones calls for further research on their nature. In particular their direct antiplatelet action (24, 25) as well as their effectiveness in the animal model of intracoronary thrombosis should be assayed.

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