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► To cite this version:

Nadji Hannachi, Laettitia Grac, Jean-Pierre Baudoin, Pierre-Edouard Fournier, Gilbert Habib, et al.. Effect of antiplatelet agents on platelet anti-staphylococcal capacity, in vitro study. International Journal of Antimicrobial Agents, 2020, pp.105890. 10.1016/j.ijantimicag.2020.105890 . hal-02445753

HAL Id: hal-02445753

<https://hal-amu.archives-ouvertes.fr/hal-02445753>

Submitted on 22 Aug 2022

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Effect of antiplatelet agents on platelet anti-staphylococcal capacity, in vitro study

Short title: Antibacterial effect of platelets.

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21 **Abstract:**

22 **Background:**

23 The involvement of platelets in anti-infectious immunity has been widely
24 demonstrated. Molecules secreted mainly by alpha granules are involved in reducing the
25 growth of certain bacterial species. However, the effect of antiplatelet treatments on the
26 platelet antibacterial ability remains poorly understood. The aim of this study was to evaluate
27 the platelet antibacterial effect against *Staphylococcus aureus*, and to evaluate the influence of
28 antiplatelet drugs on this effect.

29 **Material and methods:**

30 Blood samples were collected from healthy donors or patient treated with antiplatelet
31 therapy. Six bacterial strains of *S. aureus* were included. Bacteria were incubated with
32 platelets for four hours. Colonies were counted on blood agar. The supernatant's effect was
33 evaluated. The effect of *in vitro* antiplatelet agents and salicylic acid was also tested. **CD62P**
34 expression rate was evaluated under different conditions of infection and platelet treatment.

35 **Results:**

36 Platelets slowed the growth of the six strains of *S. aureus* ($p= 0.006$ for P6134, $p=$
37 0.001 for P6170 and P6138 and $p=0.003$ for the other strains vs bacteria alone). The
38 supernatant of platelets pre-infected with bacteria and that of platelets pre-treated with TRAP,
39 retained this antibacterial effect (Platelet-bacteria supernatant: $p= 0.018$; TRAP: $p= 0.011$ vs
40 bacteria alone). The treatment of platelets by antiplatelet drugs significantly decreased this
41 antibacterial effect (Aspirin: $p= 0.027$; Ticagrelor: $p=0.0263$; combination: $p=0.0092$ vs
42 untreated platelets). Salicylic acid also induced inhibition of this antibacterial effect ($p=0.042$
43 vs untreated platelets).

44 **Conclusion:**

45 Our study showed that antiplatelet agents decreased antibacterial effect of platelets on
46 *S. aureus*.

47 **Key words:**

48 Platelets, Bacteria, aspirin, P2Y12 inhibitors, *Staphylococcus aureus*.

49

50 **Introduction:**

51 Platelets have long been linked to hemostatic processes only [1]. To date, many
52 studies have demonstrated that platelets are involved in immunity as sentinels that are an
53 integral part of our immune system [2].

54 Platelets α granules can store hundreds of proteins. In addition to their involvement in
55 the hemostasis process, some of these molecules also play a role in anti-infectious immunity
56 [3]. This concerns thrombin Platelet Microbicidal Proteins (tPMP), such as Platelet Factor 4
57 (PF4), or thrombocidins (TC1 and 2), a carboxy-terminal diamino acid truncated versions of
58 neutrophil-activating peptide-2 (NAP-2) and connective tissue-activating peptide-III (CTAP-
59 3) respectively [4]. Staphylococcal α toxin has been shown to induce platelet degranulation,
60 releasing anti-bacterial proteins [5]. In addition, after stimulation by the same toxin, platelets
61 secrete the beta defensin 1 (HBD 1), a molecule stored in the extra granular platelet, with
62 bactericidal activity [6]. Previous studies have suggested that these proteins act by increasing
63 membrane permeability of the bacterium, leading to its death [6].

64 Antiplatelet therapy has widely shown a reduction in mortality from coronary heart
65 disease [7]. However, their influence on antibacterial effect of platelets have not been
66 thoroughly studied. The few studies conducted on this subject have shown that the secretion
67 of PMP requires the ADP pathway [8]. It has also been demonstrated that salicylic acid
68 (SAL), the main metabolite of aspirin, reduces the secretion of staphylococcal α toxin [9].
69 However, the indirect link between SAL and the secretion of platelet antibacterial proteins
70 through staphylococcal α toxin has not yet been experimentally established.

71 Although the antibacterial effect of platelets was widely described, to date, no studies
72 have evaluated the influence of antiplatelet drugs on this effect. The aim of this study was
73 therefore to analyze the platelet antibacterial effect against six strains of *Staphylococcus*

74 *aureus*, and to evaluate the influence of antiplatelet drugs such as aspirin and P2Y₁₂
75 antagonists on this effect.

76 **Material and methods :**

77 **1. Platelet preparation:**

78 Blood was drawn by venipuncture into sodium citrate from healthy subjects or from
79 patients with daily anti-platelet medication (Aspirin, P2Y₁₂ receptor antagonist or a
80 combination of both). The protocol was approved by the ethic committee of the IHU
81 méditerranée-infection (Reference 2016-002). Platelet-Rich Plasma (PRP) was prepared
82 according to the ISTH recommendations [10]. Then, platelet count determination was
83 performed using a hematology analyzer. PRP was again centrifuged at 1100g for 10 minutes
84 to obtain a platelet pellet that was suspended in Tyrode's buffer to obtain 4.10⁸ e/mL. Platelets
85 were then kept at 37°C in order to prevent activation.

86 **2. Bacterial Preparation :**

87 Six different strains of *S. aureus* from the CSUR (Collection des souches de l'unité
88 des Rickettsies, IHU Méditerranée infection, Marseille France) were used. Four of them
89 Methicillin sensitive (MSSA: P6299, P6175, P2188 and P6134) and two resistant (MRSA:
90 P6170 and P2138). These strains were previously isolated from positive blood cultures. The
91 strains were identified by Maldi Toff mass spectrometry using the Bio Typer database
92 (Bruker, Dresden, Germany). Methicillin susceptibility profiles were determined by the disc
93 diffusion method. Strains were cultured on 5% sheep blood-enriched Columbia agar (COS,
94 BioMérieux, Marcy l'Etoile, France). After 18 hours of incubation at 37°C, colonies were
95 removed and suspended in 0.9% NaCl medium to obtain the required concentrations: 1.5, 3 or
96 10 x 10⁸ CFU/ mL.

97 **3. Bacteria- platelets mixture and culturing:**

98 Bacteria and platelets were mixed at a Multiplicity of infection of 1 (except for the part
99 where a range of bacterial concentrations was tested) during 4 hours at 37 ° C under rotation
100 [6]. After four hours of incubation, the aliquots were diluted in series in Tyrode's buffer to

101 provide dilutions from 10^{-1} to 10^{-7} times the initial concentrations. Dilutions were spread on
102 COS agar (BioMérieux, Marcy l'Etoile, France). After 18 hours of incubation at 37°C ,
103 colony forming units (CFU) were counted and a comparison was made between the bacterial-
104 platelet mixture and the control consisting of the bacteria incubated with the Tyrode's buffer.
105 The percentage of growth was determined as follows: Number of CFU obtained for each
106 condition X 100 / number of CFU obtained for bacteria incubated with the Tyrode's buffer.

107 **4. Preparation of the supernatant resulting from the incubation of the bacteria with** 108 **the platelets:**

109 Platelets (4.10^8 e/mL) were incubated with *S. aureus* (P2188, strain chosen randomly,
110 3.10^8 CFU/ mL) at 37°C for one hour, allowing activation and platelet degranulation. Then,
111 the mix was centrifuged twice, the first at 1100 g for 10 minutes to remove platelets and the
112 second at 5000 g for 10 minutes to eliminate bacteria. The supernatant containing the secreted
113 platelet compounds was thus filtered using Minisart High Flow Stérile luer mâle - $0.2\ \mu\text{m}$
114 (Sartorius Stedim) to eliminate all cells. This supernatant was again incubated with the same
115 strain for a period of 4 hours at 37°C under rotation. In addition, the supernatant of resting
116 platelet and of platelets pre-activated by $10\ \mu\text{M}$ of Thrombin Receptor-Activating Peptide
117 (TRAP) (STAGO, France) were also tested.

118 **5. Effect of antiplatelet drugs *in vitro*:**

119 PRP of healthy subjects was treated with aspirin (Sanofi, Toulouse, France) at a final
120 concentration of 2 mM [11, 12], or by ticagrelor (AstraZeneca AB S-151 85 Södertälje Suède)
121 at a final concentration of $10\ \mu\text{M}$ [13], or by the combination of both drugs for 1 hour at 37°C .
122 A part of PRP remained untreated. After washing, platelets of different conditions were
123 incubated with 3×10^8 CFU/ mL of *S. aureus* (strain: P2188, strain chosen randomly). Bacteria
124 incubated in the Tyrode's buffer and bacteria incubated with untreated platelets were used as

125 controls. In addition, bacteria incubated with single drugs alone without platelets were
126 performed as a control on the interference of these drugs on bacterial growth.

127 **6. Analysis of platelet activation by flow cytometry:**

128 180 μL of treated or untreated platelets (250 G/L) were incubated with 20 μL of *S.*
129 *aureus* P2188 (10^9 CFU) for 1 hour at 37 C°. Resting and TRAP (10 μM) activated platelets
130 were used as controls. After incubation, 4 μL of Anti CD62P- PC 5 (IgG, κ monoclonal, BD
131 Biosciences) were added to 50 μL of samples and vortexed. Samples were incubated at room
132 temperature and under static conditions in the dark for 30 min, 200 μL of PBS were added
133 before analysis by flow cytometer (Beckman Coulter FC500).

134 **7. Effect of salicylic acid:**

135 Platelets of healthy subjects were incubated with 3×10^8 CFU/ mL of *S. aureus* (strain
136 P6299, strain chosen randomly) with or without SAL (Cooper, France) to a final
137 concentration of 2 mM [11, 12] according to the same protocol described above. Bacteria
138 incubated in Tyrode's buffer and bacteria incubated with untreated platelets were used as
139 controls. Also, bacteria incubated with SAL alone without platelets was performed as a
140 control on the interference of this compound on bacterial growth.

141 **8. Statistical analysis :**

142 Statistical analyzes were performed using IBM-SPSS-Statistics software version 23.
143 Comparison was made between absolute numbers of CFUs that appeared for each condition.
144 Statistical differences were evaluated using the Student Paired t test when samples were
145 normally distributed or Kruskal Wallis, Mann-Whitney U test, Wilcoxon signed ranks test for
146 samples non normally distributed with a p-value ≤ 0.05 considered to be significant.

147 **Results:**

148 **1. Platelets inhibited *S. aureus* growth:**

149 After 4 hours of incubation, the platelets significantly decreased bacterial growth of all
150 the six tested strains of *S. aureus* compared to controls. (n=11: p= 0.006 for P6134, p= 0.001
151 for P6170 and P6138 and p=0.003 for the other strains, Wilcoxon signed ranks test) (Fig 1 A).
152 The inhibition of bacterial growth by platelets remained significant whatever the
153 concentration tested compared to controls, which passed to 7.53% ± 11.7% with 1.5 x 10⁸
154 CFU/ mL (p=0.028), 6.83% ± 5.7% with 3 x 10⁸ CFU/ mL (0.027) and 13.69% ± 9.09% with
155 10⁹ CFU/ mL (p=0.028) (n= 6; Wilcoxon signed ranks test).

156 **2. Supernatant of the mix Platelet-*S. aureus* had a similar effect to that of**
157 **platelets:**

158 The supernatant of resting platelets showed no effect on bacterial growth (p = 0.682;
159 Paired t test). Interestingly, the supernatant of platelets pre-infected with bacteria and that of
160 platelets pre-activated with TRAP showed significant inhibition of *S. aureus* growth
161 compared to bacteria incubated with the Tyrode's buffer and to the supernatant of resting
162 platelets (n= 7, Platelet-bacteria supernatant: p= 0.018 and 0.0013 respectively. TRAP: p=
163 0.011 and 0.0014 respectively. Student t test) (Fig 1B).

164 **3. *In vitro* treatment by anti-platelet drugs decreased the anti-staphylococcal**
165 **effect of platelets:**

166 The treatment of platelets by antiplatelet drugs significantly decreased their
167 antibacterial effect (Fig 2A). Although the decrease in growth was significant compared to the
168 bacteria incubated with Tyrode's buffer (n=5: Aspirin: p= 0.002; Ticagrelor: p<0.001;
169 combination: p=0.0043), this decrease was significantly lower compared to that obtained with
170 the untreated platelets (Aspirin: p= 0.027; Ticagrelor: p=0.0263; combination: p=0.0092).
171 Drugs used alone, at the same concentrations as above, showed no effect on *S. aureus* growth

172 (Aspirin: p=0.256; Ticagrelor: p=0.144; combination: p=0.32; Paired t test) (Fig 2C; Supp
173 Fig 1).

174 **4. Antiplatelet drugs decreased the phenotype of platelet activation induced by *S.***
175 ***aureus*:**

176 As expected, platelets infected with *S. aureus* P2188 showed significant increase in
177 mean intensity of CD62P expression compared to untreated platelets (n=5, p=0.0009). In
178 addition, pretreatment of platelets with anti-platelet drugs before incubation with *S. aureus*
179 decreased significantly the mean intensity of CD62P expression compared to untreated
180 platelets infected with *S. aureus* (n= 5. Aspirin: p= 0.046; Ticagrelor: p= 0.0001, Association:
181 p=0.0015). Interestingly, Ticagrelor and the association of both drugs showed higher
182 inhibition rates compared to Aspirin (n=5; p= 0.003 and p=0.0378 respectively; Paired t test)
183 (Fig 3).

184 **5. *In vitro* treatment of platelets by Salicylic acid inhibited the platelet anti-**
185 **staphylococcal effect:**

186 To determine whether SAL could influence bacterial growth, the antibacterial effect of
187 platelets was tested with and without SAL. The presence of SAL significantly decreased the
188 platelet antibacterial effect compared to untreated platelets (n=5; untreated platelets:4.31% ±
189 2.6%; SAL condition:52.86% ± 29.79%; p=0.042; Wilcoxon signed ranks test). SAL, without
190 platelets, used at the same concentration showed no effect on bacterial growth (p=0.160;
191 Paired t test) (Fig 2C; Supp Fig 1).

192 **6. Platelets of patients with daily anti-platelet treatment decreased their**
193 **antibacterial effect on *S. aureus*:**

194 As shown in Fig 1A, platelets from healthy subjects significantly reduced *S. aureus*
195 P2188 growth compared to control (n=14; p= 0.001). Interestingly, platelets from patients
196 treated daily with antiplatelets exhibited a significant decrease in *S. aureus* bacterial growth

197 inhibition whatever the treatment (n= 8 for aspirin, $p < 0.001$; n=5 for P2Y₁₂ receptor
198 antagonists, $p=0.001$; n=10 for combination treatment, $p < 0.001$; Mann Whitney test) (**Fig**
199 **2B**).

200 **Discussion:**

201 We showed that platelets have an antibacterial effect on *S. aureus*. This effect was
202 bound to compounds secreted during platelet activation and was maintained even in the
203 presence of high bacterial concentrations. For the first time, our results showed that anti-
204 platelet treatment acting either on the ADP or the arachidonic acid (AA) pathways reduces
205 this antibacterial effect.

206 With regard to the overall platelet effect, we obtained a significant inhibition on all six
207 tested strains. This effect could be related to multiple types of interactions already described,
208 such as engulfment and degranulation, including both killed bacteria and those with slow
209 growth by platelet sequestration. Using supernatant fraction, the anti-bacterial effect was
210 observed only with that of activated platelets by TRAP or *S. aureus* with growth inhibition
211 comparable to that using whole platelets, suggesting that this effect was mostly the
212 consequence of platelet secretion induced by bacteria. A recent study has also shown that
213 most of the antibacterial effect of platelets was associated to their secretory capacity reporting
214 a rare observation of engulfing bacteria by platelets [14]. The exact composition of the *S.*
215 *aureus*-platelet supernatant related to antibacterial activity is not yet completely clear,
216 although we note the main molecules already reported, consisting in N-serine and N-aspartate
217 versions of PMP-1, basic peptides and derivatives, CTAP-3 [8] as well as HBD 1. The latter is
218 an extra-granular platelet peptide, also found in epithelial cells, forming part of a large group
219 of small cationic peptides (defensins), having activity against a board range of pathogens,
220 including Gram-positive and Gram-negative bacteria, by broadly targeting bacterial
221 membranes. [5, 6, 15].

222 Platelets treated *in vitro* or derived from patients under long-term antiplatelet therapy,
223 aspirin and P2Y₁₂ inhibitors, exhibited a reduced effect on bacterial growth. This suggest the
224 involvement of the two pathways in this secretory process. This was in accordance with flow

225 cytometry data in which treated platelets were associated with low level in CD62P expression
226 induced by bacteria. This therefore reflects a decrease in the spill of the α granules induced by
227 the bacteria [16]. It was shown that platelet signaling pathways leading to PMP release
228 primarily depend on P2 receptors of ADP [8]. In our study, we observed a decrease in the
229 effect with both P2Y₁₂ inhibitors and aspirin, suggesting the involvement of AA pathway also
230 in the secretion mechanism of antibacterial platelet molecules.

231 Regarding the use of aspirin and SAL *in vitro*, we confirmed the effect observed with
232 *in vivo* treated platelets and dissociated the effect of the active molecule on platelets from its
233 main metabolite. Indeed, while aspirin probably reduced this effect by inhibiting the AA
234 pathway, SAL has not yet any documented effect on platelet function. However, previous
235 studies have reported that SAL can modulate the expression of several genes regulation
236 systems in *S. aureus*, resulting, inter alia, in the inhibition of the synthesis of α toxin [9, 17].
237 The latter being involved in the secretion of platelet antibacterial peptides [5, 6]. The
238 decrease in anti-bacterial platelet effect observed with both molecules suggests that in patients
239 treated with aspirin, the attenuation of the antibacterial effect depends not only on the direct
240 pathway of AA but might also be indirectly related to the decrease in staphylococcal α toxin
241 secretion.

242 Several studies have reported a beneficial effect of antiplatelet agents in infectious
243 models such as infectious endocarditis and sepsis [18, 19]. However, other studies have
244 reported contradictory results [20]. The relationship between the infectious agent and the
245 platelets seems to be bi-directional. On one hand, the infectious agent leads to platelet
246 aggregation with further negative consequences. On the other hand, platelets act as an
247 immune actor involved in the elimination of the pathogen. In our study, we reported an
248 inhibitory effect of antiplatelet agents on the immune capacity of platelets, but we did not

249 assess their effect on hemostatic consequences in this context where antiplatelet agents may
250 retain their well-known beneficial effect.

251

252 **Conclusion:**

253 We have confirmed that platelets have an anti-staphylococcal effect and we have
254 shown for the first time that aspirin and P2Y₁₂ antagonists inhibit this effect. We also
255 demonstrated that this effect was mediated by secreted-compounds, and that the influence of
256 aspirin long-term treatment in patients on the anti-bacterial effect of derived platelets was due,
257 not only to the action of the active molecule on the platelets, but also to its main metabolite on
258 the bacteria.

259 Future studies should focus on the composition of effective supernatants generated
260 from activated platelets in order to characterize deeply each peptide or component and their
261 specific antibacterial activity.

262

263 **Declarations**

264 **Funding:** This work was supported by the Institut Hospitalo-Universitaire (IHU)
265 Méditerranée Infection, Marseille.

266 **Competing Interests:** No conflict of interest

267 **Ethical Approval:** Not required

268

269

270 **Reference:**

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331 **Figures:**

332 **Figure 1:** Secreted platelet compounds impede *S. aureus* growth:

333 (A). Effect of whole platelets on *S. aureus* growth. Values are expressed as Medians
334 and ranges. Black stars show significant differences between bacteria incubated with platelets
335 compared to bacteria incubated with Tyrode's buffer considered as 100%. n=11; **: $0.001 \leq p < 0.01$;
336 Wilcoxon signed ranks test.

337 (B). Effect of platelet supernatant on *S. aureus* growth. Values are expressed as mean
338 \pm SD. Black stars show significant differences between bacteria incubated with platelet
339 supernatants under different conditions and bacteria incubated with Tyrode's buffer. Gray
340 stars show significant differences between bacteria incubated with supernatant of pre-infected
341 or pre-activated platelets and bacteria incubated with supernatant of resting platelets. n=7; *: $0.01 \leq p < 0.05$;
342 **: $0.001 \leq p < 0.01$.; student t test.

343 **Figure 2: Platelets treated *in vitro* or obtained from subjects with daily antiplatelet**
344 **drugs have a reduced effect on bacterial growth.**

345 (A) Influence of *in vitro* treatment of platelets on their antibacterial effect. Values are
346 expressed as mean \pm SD. n=5; Paired t test.

347 (B). Effect of daily antiplatelet treatment. Values are expressed as Medians and ranges.
348 n= 8 for aspirin; n=5 for P2Y12 antagonists; n=10 for combination; Mann-Whitney Test.

349 (C). Effect of drugs used alone on bacterial growth. Values are expressed as mean \pm
350 SD. n=5; Paired t test.

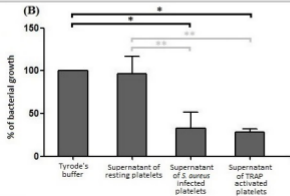
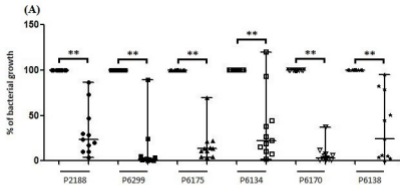
351 Grey stars depict the statistical differences between antiplatelet conditions compared
352 to untreated platelet condition. Black stars depict statistical differences between treated or
353 untreated platelet conditions compared to the bacteria incubated with Tyrode's buffer. NS:
354 Not significant difference; *: $0.01 \leq p < 0.05$; **: $0.001 \leq p < 0.01$ and ***: $p < 0.001$.

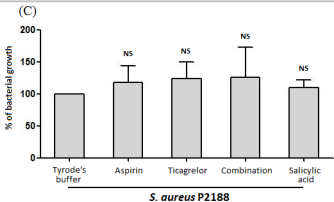
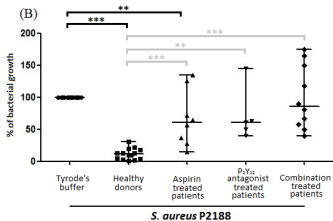
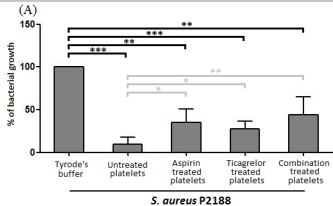
355 **Figure 3: Antiplatelet agents decreased the platelet CD62P expression induced by**
356 **bacteria.**

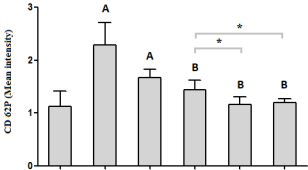
357 (1) untreated, (2) Aspirin, (3) Ticagrelor and (4) combination (Ticagrelor + Aspirin).
358 Median fluorescence intensity (MFI) of expressed CD62P are shown. Values are expressed as
359 mean \pm SD. (A) shows significant difference on CD62P mean intensity compared to untreated
360 platelets. (B) shows significant difference on CD62P mean intensity compared to bacteria-
361 untreated platelets. Black stars show significant difference between the different treatment
362 conditions. n=5. p<0.05. Paired t test.

363 **Supplementary figure 1:**

364 (A) Growth curve of *S. aureus* P2188 growth in trypticase soy agar (TSB), in the
365 absence of SAL and in the presence of SAL at doses of: 1mM, 2mM, 5mM, 10mM, and
366 20mM. No apparent difference was perceived until the concentration of 20 mM. (B) Growth
367 curve of *S. aureus* P2188 growth in TSB, in the absence of aspirin and in the presence of
368 aspirin at doses of: 1mM, 2mM, 5mM, 10mM, and 20mM. No apparent difference was
369 perceived until the concentration of 20 mM. (C) Growth curve of *S. aureus* P2188 growth in
370 TSB, in the absence of ticagrelor and in the presence of ticagrelor at doses of: 2.5 μ M, 5 μ M,
371 10 μ M, 20 μ M and 30 μ M. No apparent differences were perceived with all the concentrations
372 tested.







| | | | | | | |
|------------------|---|------|---|-----|-----|-----------|
| <i>S. aureus</i> | - | - | + | + | + | + |
| Drugs | | TRAP | - | ASA | TCG | ASA + TCG |