In this study the question was addressed whether cAMP mediated drugs induce a differential reduction of branches of the arachidonic acid metabolism rather than a global reduction of eicosanoid synthesis. The isolated lungs of actively sensitized rats were employed to study prostaglandin and leukotriene release in the presence and absence of the cAMP mediated drugs theophylline, milrinone, sulmazole, isobutyl-methylxanthine and salbutamol. The release of eicosanoids as measured by RIA was predominantly basal and continuous, with a mild antigen induced stimulation only for TXB2 and the leukotrienes. All drugs reduced eicosanoid release globally. It is concluded that cAMP mediated drugs interfere with arachidonic acid metabolism at a site proximal to the branching into lipoxigenase and cyclo-oxygenase pathways.

**Key words:** AA 861, cAMP, Leukotrienes, Lung, Milrinone, Prostaglandins

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**Introduction**

Drugs that reduce eicosanoid synthesis might be valuable in symptomatic and curative therapy of various inflammatory diseases. Such drugs include cAMP elevating compounds like the xanthine derivatives theophylline and IBMX, and β-sympaticomimetic drugs and inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. Milrinone and sulmazole are two recently developed cardiotonic and vasodilator drugs that belong to the latter category. In previous experiments we compared the effects of milrinone, sulmazole and theophylline in the rat isolated lung. We showed that these drugs inhibited antigen induced bronchoconstriction, vasoconstriction and SRS-A release, leaving the release of the preformed mediators histamine and serotonin unaffected. Rossing *et al.* also showed a bronchorelaxant effect of milrinone against a variety of agonists in trachea, lung parenchyma and pulmonary artery, that could be counteracted by indomethacin. This led them to hypothesize that milrinone exerted a differential effect on arachidonic acid metabolism and either induced the synthesis of bronchorelaxing prostaglandins or reduced indomethacin enhanced production of bronchoconstrictive leukotriens. In this study we addressed the question of whether cAMP mediated drugs induce a differential reduction of branches of the arachidonic acid metabolism rather than a global reduction of eicosanoid synthesis. We incorporated AA 861 in the study as a selective inhibitor of leukotriene synthesis to check whether selective modulation of arachidonic acid metabolism could be shown in our model.

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**Materials and Methods**

**Sensitization:** Male Wistar rats, weighing 180–200 g, were actively sensitized. For this purpose, an emulsion was made of trinitrophenylovalbumin in aluminium phosphate, containing 50 µg ml⁻¹ TNP-OVA and 6 mg/ml AlPO₄. Of this solution, 2 ml was injected intraperitoneally. After 12–21 days the animals were used for experiments.

**Rat isolated and ventilated lungs with vascular perfusion:** Lungs were removed under pentobarbitone-Na anaesthesia (50 mg kg⁻¹ i.p.), suspended in a Plexiglas chamber and perfused through the pulmonary artery with Krebs-Ringer solution (37°C and gassed with 5% CO₂ in O₂) at a rate of 7.5 ml min⁻¹. The lungs were ventilated (25 min⁻¹) with a Braun piston pump by applying vacuum within the surrounding chamber, alternating from −6.5 cm H₂O to −1 cm H₂O. A constant flow of O₂ with 5% CO₂ into the trachea imposed a positive pressure to the trachea, which was kept constant at 5.5 cm H₂O, by means of an adjustable
water seal connected with a T-piece to the inflation tube. Thus, the end expiratory pressure was constant, while the end inspiratory pressure rose as a function of airway diameter. The area under the end inspiratory pressure curve was measured and expressed as a percentage of the area under the (horizontal) curve representing the maximally attainable end inspiratory pressure. Pulmonary artery pressure and tracheal pressure were measured by means of an Ailtech (MEDIO-E) and an Elema-Schönander transducer, respectively. Each pair of lungs was allowed to equilibrate for 15 min. After antigen challenge, perfusate was continuously collected for a period of 7 min and analysed for mediators. In one series of experiments, designed to study the influence of antigen challenge on eicosanoid release, a triplet of 5 min perfusate samples was collected: one before antigen challenge, a second from 1 to 6 min after antigen challenge and a third from 10 to 15 min after challenge.

Antigen challenge and drug treatment: Lungs were challenged with a bolus injection of 0.2 ml (50 μg ml⁻¹) undiluted TNP-OVA solution, into the pulmonary artery. This proved to be a supramaximal dose in previous studies. All drugs were added to the perfusion fluid during the entire course of the experiments starting at least 15 min before antigen challenge. The drugs were either dissolved in the perfusion fluid or added to the infusion fluid just prior to the entrance into the pulmonary artery.

Determination of mediators: Histamine was measured fluorometrically after pre-column derivatization with o-phthalaldehyde and subsequent separation by means of HPLC. All eicosanoids were measured by RIA. For this purpose, perfusate was collected in 1 min fractions, from 1 to 7 min after antigen challenge. Each fraction was applied to a Sep-Pak C₁₈ cartridge. The cartridges had been prewashed with ethanol, water and, finally, 0.5% EDTA in water (pH = 5.5). Prior to elution with 4 ml ethanol, the cartridges were flushed with 5 ml water. [³H]-PGA₂ was used as an internal standard to determine recovery. Samples of 50 or 100 μl were dried and dissolved in RIA buffer. Detection ranges were: 5-HETE (0-500 ng), 12-HETE (0-1000 ng), 15-HETE (0-250 ng), PGE₂ (0-1000 ng), 6k-PGF₁₅ (0-250 ng), PGF₂α (0-500 ng), TXB₂ (0-250 ng), LTD₄ (0-500 ng) and peptidoleukotrienes (0-500 ng). The detection limits for the prostaglandins, peptidoleukotrienes and HETEs were 2, 5 and 10 pg respectively.

Cross reactivities for radioimmunoassays of LTs, HETEs and prostaglandins (% at 50% B/B₀), were less than 1%, except for anti-5-HETE with LTD₄ (4%), anti-PGF₂α with 6k-PGF₁₅ (2.2%) and anti-PGF₂α with PGE₂ (1.3%). The peptidoleukotrienes assay discriminates rather poorly between LTC₄, LTD₄, LTE₄ or LT₄, cross-reactivities with these LTs being 55%, 100%, 51% and 67% respectively.

Drugs and materials: Antisera and standards for RIAs were obtained from Advanced Magnetics Inc., (Cambridge, MA, USA) and [³H]-labelled antigen from the Radiochemical Centre, (Amersham, UK). Ovalbumin, grade V, was purchased from Sigma. Trinitrobenzenesulphonic acid was obtained from Baker Chem. (Deventer, The Netherlands). Sulmazole and milrinone were gifts from Dr Karl Thomae GmbH (Biberach, Germany) and Sterling-Winthrop (USA), respectively. AA 861 (2-12-hydroxy-docosa-5,10 -dienyl - 3,5,6 - trimethyl - 1,4 - benzoquinone) was supplied by Takeda Chemical Industries Ltd (Osaka, Japan). Theophylline, salbutamol and isobutylmethylxanthine (IBMX) were all purchased from Sigma. Sulmazole, theophylline, salbutamol and IBMX were dissolved in the perfusion medium, while milrinone and AA 861 were administered with an infusion to the perfusion medium. For this purpose, milrinone was dissolved in Krebs acidified with hydrochloric acid to a pH of 4.5, not affecting the pH of the perfusion fluid. AA 861 was dissolved in DMSO (final concentration 0.3%). All constituents of the perfusion fluid, ethanol and o-phthalaldehyde were obtained from Merek, and were of analytical grade. Sep-Pak C₁₈ cartridges were purchased from Millipore Co.

Data analysis: In the text and figures, data represent the arithmetic means ±SE. Mediator release was expressed as total amount present in the perfusate collected from 1 to 7 min after challenge (yielding 45 ml perfusate). Three control groups were incorporated in this study, one matched with the theophylline 10 and 100 μM, milrinone and sulmazole treated groups, a second matched with the theophylline 1 mM, IBMX and salbutamol group and a third DMSO 0.3% control group. In the second control group, time dependency of mediator release was studied by collecting and analysing 1 min fractions separately, again from 1 to 7 min after challenge. The sum of these fractions were used for comparison with the drug treated groups (theophylline 1 mM, IBMX, salbutamol). Differences in mean bronchoconstriction and mediator release were evaluated by means of a one-way analysis of variance on raw data, comparing the absolute figures from treated groups with their respective control groups. If the F-test showed significant differences between groups (p < 0.05), differences in means were evaluated with Duncan's multiple range test and judged to be significant if p < 0.05.
Results

Time dependency of mediator release after antigen challenge:
(a) Preformed mediators. Antigen challenge induced a rapid and substantial release of histamine up to $857 \pm 105 \text{ ng min}^{-1}$, $n = 7$ (Fig. 1a). A total of approximately $2000 \text{ ng histamine}$ was released by each pair of lungs (weighing approximately 1 g). Within 1 min after challenge, maximal concentrations of histamine were measured. After 1 min histamine release gradually subsided, reaching basal release ($227 \pm 43 \text{ ng min}^{-1}$, $n = 7$) in 7 min. (b) Eicosanoids. The mono-HETEs, 5-HETE, 12-HETE and 15-HETE, were synthesized and released by the rat isolated lung. Of these, the release of 12-HETE was the most predominant (Fig. 1b). 15-HETE and 5-HETE were released in small amounts only ($\pm 500 \text{ pg min}^{-1}$) (Fig. 1c).

PGI$_2$, measured as 6k-PGF$_2$ was the predominant bronchorelaxant prostaglandin. Neither PGI$_2$, nor PGE$_2$ showed a clear release pattern after antigen stimulation (Fig. 1d).

The bronchoconstrictive prostaglandin PGF$_{2\alpha}$ and the stable metabolite of thromboxane A$_2$, TXB$_2$, were released in equal amounts ($\pm 1000 \text{ pg min}^{-1}$) (Fig. 1e). The release of TXB$_2$ was stimulated significantly at 2 min after antigen challenge, declining thereafter.

LTB$_4$ and the peptidoleukotrienes, were released in relatively small amounts by the rat lung (Fig. 1f). LTB$_4$ showed a clear release pattern after antigen stimulation reaching its maximum at $t = 3 \text{ min.}$

![Graphs of mediator release over time](image-url)
Peptidoleukotriene release was also stimulated, with a maximal release at \( t = 4 \) min.

In four separate experiments, we attempted to establish a release pattern for the peptidoleukotrienes, 12-HETE and TXB\(_2\), by collecting a triplet of 5 min perfusate fractions. The results are shown in Fig. 2. A stimulatory release pattern could be observed for the peptidoleukotrienes and TXB\(_2\). Antigen challenge in the rat lung did not stimulate the release of 12-HETE and 6k-PGF\(_{1a}\).

For all eicosanoids, antigen-induced synthesis was low and in most instances undistinguishable from basal release (Figs 1 and 2). In the experiments where modulation of mediator release by drugs was studied, total, i.e., basal and antigen induced release, was measured.

Modulation of mono-HETE production by the drugs: The mono-HETEs were quite uniformly affected by theophylline, milrinone and sulmazole (Table 3). These drugs reduced mono-HETE production at high concentrations only, with the exception of 15-HETE, the production and release of which seemed to be resistant to sulmazole. Salbutamol, IBMX and AA 861 reduced 5-HETE, but not 12-HETE production.

Bronchoconstriction and total mediator release by antigen challenged lungs: Table 1 lists the results of antigen induced bronchoconstriction and total mediator release in the three control groups (\( n = 6 \)). As mentioned, we performed the experiments described here in different seasonal periods and therefore had two different control groups and one vehicle control group. No significant differences in mediator release between these groups were observed, except for 5-HETE and LTB\(_4\). In control group 2, PGF\(_2\alpha\) measurement failed.

Modulation of antigen induced bronchoconstriction and total histamine release: In Table 2 the dose dependent inhibition of antigen induced bronchoconstriction by theophylline, milrinone, sulmazole, IBMX and salbutamol is depicted. AA 861 at a concentration of 5 \( \mu \)M did not affect antigen induced bronchoconstriction. Histamine release was rather insensitive to the action of various drugs. Only sulmazole at a concentration of 100 \( \mu \)M, reduced the release of this autacoid (Table 2). Theophylline 1 mM was not

| Table 1. Bronchoconstriction and mediator release in control groups |
|---------------------------------------------------------------|
|                     | Control 1 | Control 2 | Control 3 |
|---------------------|-----------|-----------|-----------|
| AUC (%)             | 32.0 ± 3.8| 28.9 ± 4.8| 30.7 ± 4.8|
| Histamine (ng)      | 1774 ± 178| 2399 ± 362| 2294 ± 437|
| 5-HETE (ng)         | 3.0 ± 0.4 | 4.7 ± 0.1*| 3.6 ± 0.6 |
| 12-HETE (ng)        | 26.4 ± 2.1| 25.6 ± 1.4| 26.9 ± 6.2|
| 15-HETE (ng)        | 1.2 ± 0.1 | 1.8 ± 0.3 | 1.5 ± 0.4 |
| PGE\(_2\) (ng)      | 22.6 ± 5.4| ND        | 20.8 ± 2.1|
| 6k-PGF\(_{1a}\) (ng)| 60 ± 18   | 40 ± 11   | 26.1 ± 2.9|
| PGF\(_2\alpha\) (ng)| 7.0 ± 2.0 | 7.5 ± 1.0 | 5.9 ± 0.9 |
| TXB\(_2\) (ng)      | 5.4 ± 0.6 | 5.7 ± 0.9 | 4.6 ± 0.9 |
| LTB\(_4\) (ng)      | 12.1 ± 1.9| 3.1 ± 0.8*| 11.6 ± 1.4|
| PLTs (ng)           | 3.5 ± 0.2 | 4.8 ± 1.0 | 2.9 ± 0.4 |

Control groups were studied at different periods. Although the lungs were challenged with antigen, total (antigen induced and basal) mediator release is depicted (means ±SE). The asterisks indicate values that are significantly different from the values in the other two groups (\( p < 0.05, n = 6 \)). ND: not determined. AUC: area under curve, bronchoconstriction; PLTs: peptidoleukotrienes.

| Table 2. Modulation of total histamine release and antigen induced bronchoconstriction |
|-----------------------------------------------|
| Drug               | AUC (%) | HR (%) |
|---------------------|---------|--------|
| Theophylline 0.01 mM| 24 ± 13*| -5 ± 25|
| 0.1 mM              | 29 ± 12*| 27 ± 19|
| 1 mM                | 73 ± 6**| ND     |
| Milrinone 0.01 mM   | 25 ± 11*| -2 ± 16|
| 0.1 mM              | 50 ± 8**| 21 ± 18|
| Sulmazole 0.01 mM   | 31 ± 10*| 31 ± 10|
| 0.1 mM              | 72 ± 8* | 33 ± 10*|
| IBMX 10 \( \mu \)M | 54 ± 8**| 12 ± 15|
| Salbutamol 1 \( \mu \)M | 11 ± 18 | 8 ± 14|
| 10 \( \mu \)M       | 36 ± 10*| 15 ± 16|
| AA 861 5 \( \mu \)M | 13 ± 11 | 21 ± 10|

Data are expressed as percentage inhibition of control data of bronchoconstriction and histamine release as given in Table 1, and represent means ±SEM, \( n = 6 \) for all (the SEM is calculated from the variances of both the control and the treated values). The asterisks indicate significance of difference with controls at \( p < 0.05 \) or, if double, at \( p < 0.01 \). AUC: area under curve; HR: histamine release; ND: not determined.
Modulation of total eicosanoid release

| Drug       | 5-HETE | 12-HETE | 15-HETE | PGE2  | 6PGF_{1a} | PGF_{2a} | TXB2 | LTB4 | pepLT |
|------------|--------|---------|---------|-------|-----------|----------|------|------|-------|
| Theophylline |        |         |         |       |           |          |      |      |       |
| 0.01 mM    | 0      | 7 ± 12  | 12 ± 13 | 79 ± 8' | 61 ± 16*  | 27 ± 24  | 1 ± 7  | 24 ± 13 | 0     |
| 0.1 mM     | 7 ± 20 | 3 ± 13  | 29 ± 7'  | 86 ± 5'  | 80 ± 6*   | 49 ± 15* | 22 ± 17 | 43 ± 13*  | 0     |
| 1 mM       | 76 ± 3' | 40 ± 12* | 83 ± 6*  | ND     | 75 ± 7   | 62 ± 6'  | 74 ± 5' | 82 ± 8*  | 87 ± 4* |
| Milrinone   |        |         |         |       |           |          |      |      |       |
| 0.01 mM    | 0      | 23 ± 11 | 17 ± 13 | 82 ± 7* | 80 ± 7*   | 47 ± 15* | 34 ± 11* | 29 ± 19 | 0     |
| 0.1 mM     | 31 ± 11* | 29 ± 8'  | 37 ± 12* | 86 ± 8*  | 80 ± 6*   | 54 ± 14* | 76 ± 5* | 66 ± 7*  | 35 ± 5* |
| Sulmazole  |        |         |         |       |           |          |      |      |       |
| 0.01 mM    | 28 ± 14 | 6 ± 10  | 26 ± 9   | 56 ± 22* | 74 ± 10*  | 51 ± 16* | 35 ± 8* | 44 ± 12* | 22 ± 12 |
| 0.1 mM     | 52 ± 7*  | 53 ± 5*  | 26 ± 15  | 96 ± 4*  | 87 ± 5*   | 82 ± 5*  | 89 ± 3* | 83 ± 4*  | 51 ± 9* |
| Salbutamol |        |         |         |       |           |          |      |      |       |
| 1 μM       | 66 ± 2' | 36 ± 13  | ND      | ND     | 58 ± 13*  | 67 ± 7*  | 64 ± 10* | 32 ± 20 | 78 ± 6* |
| 10 μM      | 57 ± 5'  | ND      | ND      | ND     | 66 ± 10*  | 66 ± 5*  | 56 ± 13* | 20 ± 33 | 73 ± 9* |
| IBMX       |        |         |         |       |           |          |      |      |       |
| 10 μM      | 76 ± 2*  | 6 ± 21   | ND      | ND     | 81 ± 5*   | 67 ± 5*  | 59 ± 8* | 48 ± 19* | 70 ± 6* |
| AA 861     |        |         |         |       |           |          |      |      |       |
| 5 μM       | 65 ± 9'  | 4 ± 25   | 66 ± 10* | 90 ± 3' | 30 ± 19   | 43 ± 12* | 20 ± 21 | 99 ± 2*  | 73 ± 3' |

Data are expressed as percentage inhibition of control data of bronchoconstriction and mediator release as given in Table 1 (theophylline 0.01 and 0.1 mM, milrinone and sulmazole, control 1; theophylline 1 mM, salbutamol and IBMX, control 2; AA 861, control 3) and represent means ±SE (the SE is calculated from the variances of control and treated values). The asterisks indicate significant differences with controls at p < 0.05 or, if double, at p < 0.01. One-way analysis of variance was performed on raw data, not on percentages. '0' is entered whenever the calculated percentage of inhibition was negative, without being significantly different from zero. In no case was a potentiation of arachidonic acid metabolism observed. ND: not determined.

evaluated, since the aminophylline used for these high concentrations interfered with the fluorometric detection of histamine.

Modulation of bronchorelaxant prostaglandins: The production of the bronchorelaxant prostaglandin 6k-PGF_{1a} was inhibited by five of the six drugs tested. AA 861 had no effect (Table 3). For sulmazole, salbutamol and theophylline obvious dose dependency was observed, while milrinone apparently had reached its maximal effect at 0.01 mM. PGE_{2} showed a pattern similar to 6k-PGF_{1a}. Since PGE_{2} data fail in the second control group, the effect of theophylline 1 mM, IBMX, salbutamol could not be calculated. However, in absolute figures, the amounts of PGE_{2} were comparable to the other treated groups, ranging from 1.7 to 5.3 ng, as opposed to 22.6 ng in control group 1 (Table 2). Surprisingly, PGE_{2} production was strongly reduced in the AA 861 treated group.

Modulation of bronchoconstrictive prostaglandins: Production of PGF_{2a} and TXB_{2} was effectively reduced by all cAMP mediated drugs (Table 3). For theophylline, milrinone and sulmazole a dose dependency was observed. Increasing the dose of salbutamol from 1 to 10 μM had no additional effect on leukotriene production. AA 861 caused a moderate reduction of PGF_{2a}, leaving TXB_{2} synthesis unaffected.

Modulation of leukotriene production: Synthesis of LTB_{4} and the peptidoleukotrienes were reduced by theophylline and milrinone at high concentrations. Sulmazole and IBMX already at 0.01 mM reduced LTB_{4} production significantly (Table 3). AA 861 almost abolished LTB_{4} and peptidoleukotriene synthesis. In contrast, salbutamol had no affect on LTB_{4} synthesis.

Discussion

In this paper we confirmed and extended earlier observations on the reduction of synthesis and release of peptidoleukotrienes by milrinone, sulmazole and theophylline in the rat isolated lung. Rossing and coworkers postulated a selective induction of bronchorelaxant prostaglandins by milrinone to explain the inhibition of milrinone induced bronchorelaxation by indomethacin. However, our data show that these and other supposedly cAMP mediated drugs reduce arachidonic acid metabolism in a global rather than a differential manner.

Global reduction of arachidonic acid metabolism by cAMP mediated drugs suggests interference with an early stage of arachidonic acid metabolism prior to separation into the lipoxygenase and cyclooxygenase pathway. This might be explained by an evaluation of intracellular cAMP reducing the release of arachidonic acid from the phospholipid pool. It has been shown for instance that beta agonists, by stimulation of adenylate cyclase, reduce phospholipase A_{2} activity and the subsequent release of arachidonic acid metabolites. In accordance with other reports, histamine release was not affected by the drugs tested. 5,11,12
Apparentlly, production of histamine release is less susceptible to elevation of cAMP than eicosanoid production.\(^1,\)\(^3,\)\(^13\)

For theophylline, milrinone and sulmazole, mechanisms of action other than cAMP elevation have been proposed. Antagonism of adenosine receptors is the most strongly advocated alternative. On the basis of previous experiments we argued against adenosine antagonism as a likely mechanism of action for milrinone and sulmazole in the concentrations used in this study.\(^14\)

Milrinone is a selective inhibitor of the phosphodiesterase enzyme complex III,\(^3\) which hydrolyses mainly cAMP. Whether this selectivity still holds in the rat lung for concentrations of 0.01 to 0.1 mM is uncertain. In view of the similarity of the pharmacological profiles of theophylline, IBMX, sulmazole and theophylline it is likely that nonselective inhibition of the cyclic nucleotide phosphodiesterase isozyme complex is responsible for the observed effects.

Using RIA, a wide spectrum of eicosanoids could be measured in the perfusate of the isolated rat lungs, comprising major representatives of the two most important pathways of arachidonic acid metabolism. Of the mono-HETEs, 12-HETE was the most predominant, whereas only small amounts of 15- and 5-HETE were produced. The same holds for guinea-pigs, whereas in man, 15-HETE production is more pronounced.\(^15\) The quantities of arachidonic acid metabolites measured in our experiments, are largely correlated with those found in rat mast cells, human lung parenchyma and the guinea-pig isolated lung.\(^15,\)\(^17\) TXB\(_2\) and leukotrienes however seem to be more prominent in the guinea-pig than in the rat. The production of histamine, TXB\(_2\), LT\(_B_4\) and the peptidoleukotrienes was stimulated by antigen, as was also shown by Turner and Dollery (1988), using the isolated guinea-pig lung.\(^18\) In contrast, no clear effect of antigen induced bronchoconstriction and the production of eicosanoids in isolated rat lungs.\(^9,\)\(^13\) Arachidonic acid metabolism is impeded at a site proximal to the separation into lipoxygenase and cyclooxygenase pathways. Whether differences in this respect exist between phosphodiesterase inhibitors and stimulators of adenylate cyclase, remains to be established. The results with AA 861 show that immediate antigen induced bronchoconstriction is not mediated by leukotrienes. Finally, we suggest that AA 861 might not in all conditions be a selective 5-lipoxygenase inhibitor.

In conclusion, all cAMP mediated drugs reduced antigen induced bronchoconstriction and the production of eicosanoids in isolated rat lungs. Arachidonic acid metabolism is impeded at a site proximal to the separation into lipoxygenase and cyclooxygenase pathways. Whether differences in this respect exist between phosphodiesterase inhibitors and stimulators of adenylate cyclase, remains to be established. The results with AA 861 show that immediate antigen induced bronchoconstriction is not mediated by leukotrienes. Finally, we suggest that AA 861 might not in all conditions be a selective 5-lipoxygenase inhibitor.

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