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Article

# Inhibition of Enzymatic Acetylation-Mediated Resistance to Plazomicin by Silver Ions

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**Abstract:** Plazomicin is a recently U.S. Food and Drug Administration (FDA)-approved semisynthetic aminoglycoside. Its structure consists of a sisomicin scaffold modified by adding a 2(S)-hydroxy aminobutyryl group at the N1 position and a hydroxyethyl substituent at the 6' position. These substitutions produced a molecule refractory to most aminoglycoside-modifying enzymes. The main enzyme within this group that recognizes plazomicin as substrate is the aminoglycoside 2'-N-acetyltransferase type Ia [AAC(2')-Ia], which reduces the antibiotic's potency. Designing formulations that combine an antimicrobial with an inhibitor of resistance is a recognized strategy to extend the useful life of existing antibiotics. We have recently found that several metal ions inhibit acetylation of numerous aminoglycosides catalyzed by the aminoglycoside 6'-N-acetyltransferase type Ib [AAC(6')-Ib]. In particular,  $\text{Ag}^{1+}$ , which also enhances the effect of aminoglycosides by other mechanisms, is very effective in interfering with AAC(6')-Ib-mediated resistance to amikacin. Here we report that silver acetate is a potent inhibitor of AAC(2')-Ia-mediated acetylation of plazomicin *in vitro*, and it reduces resistance levels of *Escherichia coli* carrying *aac(2')-Ia*. The resistance reversion assays produced equivalent results when the structural gene was expressed under the control of the natural or the *blatem-1* promoters. The antibiotic effect of plazomicin in combination with silver was bactericidal, and the mix did not show significant toxicity to human embryonic kidney 293 (HEK293) cells.

**Keywords:** AAC(2')-Ia; aminoglycoside 2'-N-acetyltransferase type Ia; aminoglycoside; multidrug resistance; metal ions; plazomicin; adjuvant

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## 1. Introduction

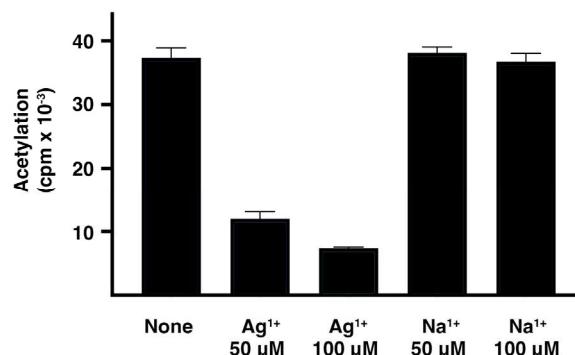
Nosocomial- and community-acquired pathogens have become resistant to many different antibiotics, and in some cases, they are virtually untreatable [1]. Numerous Enterobacterales clinical isolates possess genes coding for aminoglycoside modifying enzymes, extended-spectrum  $\beta$ -lactamases, and carbapenemases [2, 3]. Colistin remains an option for treating life-threatening multidrug-resistant infections caused by some of these bacteria [4]. However, resistant variants have already been found in several geographical regions and may soon become prevalent [5, 6]. Other novel options include ceferidoreol and new  $\beta$ -lactams/ $\beta$ -lactamase inhibitors [7-9]. Aminoglycosides are excellent tools for treating a wide variety of Gram-negative and Gram-positive infections. Unfortunately, the rise and dissemination of aminoglycoside modifying enzymes the major mechanism of resistance to these antibiotics in the clinical setting, have reduced their effectiveness [10-12]. Therefore, developing new antibiotics or therapeutic strategies is necessary to generate viable treatment options [13]. Numerous analogs to natural aminoglycosides have been designed to resist the action of resistance enzymes. Many of these compounds, known as semisynthetic aminoglycosides, are refractory to the action of most aminoglycoside modifying enzymes and have been successfully introduced in the clinical setting to treat resistant infections [14, 15]. Plazomicin is a next-generation semisynthetic aminoglycoside designed modifying sisomicin by the addition of a

2(S)-hydroxy aminobutyryl group at the N1 position and a hydroxyethyl substituent at the 6' position [16, 17]. These modifications result in a molecular structure refractory to most aminoglycoside modifying enzymes [10, 18-21]. Plazomicin was approved in 2018 by the FDA to be used in patients with limited or no options for alternative treatment. It is active against multidrug resistant Enterobacteriales, including strains producing carbapenemases and extended-spectrum  $\beta$ -lactamases, while showing tolerable levels of nephrotoxicity and ototoxicity [19, 22, 23]. Unfortunately, despite the substitutions that make plazomicin a non-substrate for most aminoglycosides modifying enzymes, the AAC(2')-Ia enzyme identified in the chromosome of *Providencia stuartii*, can catalyze the inactivation of the antibiotic molecule through the transfer of an acetyl group from acetyl-CoA to the 2'-N-position [24, 25]. Although this enzyme is not usually found in clinical isolates, it is a matter of time before it disseminates and becomes prevalent if the use of plazomicin increases. An obvious path to deal with the rise and dissemination of aminoglycoside modifying enzymes is the continuous design of semisynthetic aminoglycosides. However, design of new generations of semisynthetic aminoglycosides has proven costly and time consuming. These stumbling blocks warrant exploring alternative strategies such as the development of inhibitors of the enzymatic inactivation that together with the aminoglycoside form a combination therapy effective against resistant pathogens [13]. The recent finding that metal ions, some of them in complex with ionophores, inhibit the AAC(6')-Ib-catalyzed acetylation of aminoglycosides and induce a reduction in the minimal inhibitory concentration (MIC) of amikacin to susceptibility levels proved this concept and increased expectations that multidrug resistant infections could be treated by these combination therapies [26-31]. In particular,  $\text{Ag}^{1+}$  inhibits the acetylation of amikacin by AAC(6')-Ib and reverses resistance in bacteria in culture at low concentrations without needing an ionophore [31]. This article describes the inhibition of AAC(2')-Ia-mediated plazomicin-resistance by  $\text{Ag}^{1+}$  in *Escherichia coli* harboring a recombinant clone containing the *aac(2')-Ia* gene.

## 2. Results

### 2.1. Effect of $\text{Ag}^{1+}$ on AAC(2')-Ia-mediated acetylation of plazomicin

$\text{Ag}^{1+}$  drastically interfered with the acetylation of plazomicin (Figure 1). The presence of silver acetate in the reaction mixture produced significant inhibition while the addition of sodium acetate did not produce any changes in acetylation levels. These results, taking together with previous research showing that metal ions have the capability to inhibit the enzymatic acetylation of aminoglycosides, are an encouraging indication that  $\text{Ag}^{1+}$  could serve as an adjuvant to plazomicin if AAC(2')-Ia or a similar enzyme disseminates among bacterial pathogens.



**Figure 1.** Effect of  $\text{Ag}^{1+}$  on (2')-Ia activity. Assays were performed in triplicate by the phosphocellulose paper binding method with soluble extracts obtained from *E. coli* TOP10(pUC57AAC2Ia) cells, the reaction mixture containing 200 mM Tris HCl pH 7.6 buffer, 0.25 mM  $\text{MgCl}_2$ , 330  $\mu\text{M}$  plazomicin, the indicated concentrations of silver acetate ( $\text{Ag}^{1+}$ ) or sodium acetate ( $\text{Na}^{1+}$ ), and 0.05  $\mu\text{Ci}$  of [ $\text{acetyl-14C}$ ]-acetyl-coenzyme A (specific activity 60  $\mu\text{Ci}/\mu\text{mol}$ ) in a final volume of 30  $\mu\text{l}$ .

## 2.2. Effect of Ag<sup>1+</sup> on AAC(2')-Ia-mediated resistance to plazomicin

The *E. coli* TOP10(pUC57AAC2Ia) MIC of plazomicin, as determined using commercial E-strips, was 12 µg/ml. To assess the effect of Ag<sup>1+</sup> on the resistance to plazomicin of growing cells, *E. coli* TOP10(pUC57AAC2Ia) was cultured in the presence of silver acetate in addition to plazomicin. A reduction in the level of growth was observed when only plazomicin was present (Table 1). However, the cells grew to an OD<sub>600</sub> consistent with heavy growth, confirming that AAC(2')-Ia confers substantial resistance to the antibiotic. Addition of silver acetate at 4 µM was sufficient to completely inhibit growth in the presence of plazomicin at a sub-MIC concentration (4 µg/ml) (Table 1). Furthermore, when the concentration of plazomicin was 8 µg/ml, still a sub-MIC value, 2 µM silver acetate was enough to inhibit growth (Table 1). A control experiment adding sodium acetate showed no differences in growth in the presence or absence of the addition (Table 1). The results described in this section unequivocally indicated that Ag<sup>1+</sup> interferes with resistance to amikacin mediated by AAC(6')-Ib.

**Table 1.** Growth in the presence of plazomicin and silver acetate.

Plazomicin (µg/ml)	Silver Acetate (µM)				Sodium Acetate (µM)	
	OD <sub>600</sub>				OD <sub>600</sub>	
0	1	2	4	0	8	
0	3.21 ± 0.02	3.09 ± 0.01	3.09 ± 0.02	3.18 ± 0.11	3.04 ± 0.06	3.12 ± 0.08
4	3.05 ± 0.08	2.86 ± 0.11	1.95 ± 0.01	0.12 ± 0.01	1.39 ± 0.03	1.34 ± 0.06
8	1.21 ± 0.01	1.26 ± 0.01	0.10 ± 0.03	0.02 ± 0	1.13 ± 0.02	1.08 ± 0.04

Cultures of *E. coli* TOP10(pUC57AAC2Ia) were performed in cation-adjusted Mueller Hinton with the indicated additions.

Studies on the expression of the aac(2')-Ia gene suggest that in its natural location, the *P. stuartii* chromosome, is subjected to regulation [32]. To discard any regulatory role in the action of Ag<sup>1+</sup>, the AAC(2')-Ia open reading frame was placed downstream of the blatem-1 promoter and cloned using pUC57 as cloning vector to generate the recombinant plasmid pUC57PBLAAC2Ia. The MIC of plazomicin of *E. coli* TOP10(pUC57PBLAAC2Ia) was 12/16 µg/ml. Table 2 shows that the results of this experiment were similar to those observed with the gene under the control of the natural promoter. The resistance levels in liquid medium were slightly lower with the gene that carries the blatem-1 promoter. However, inhibition of resistance by Ag<sup>1+</sup> was identical with both promoters.

**Table 2.** Growth in the presence of plazomicin and silver acetate.

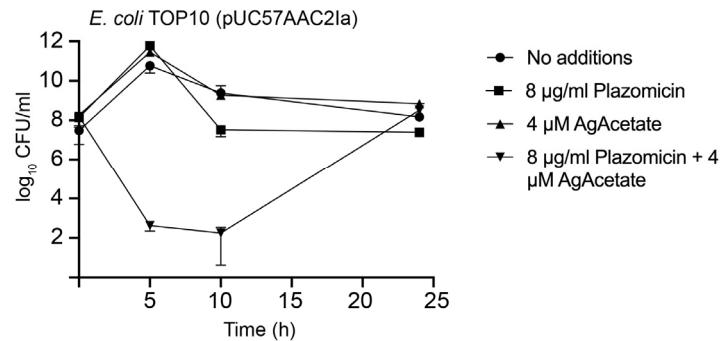
Plazomicin (µg/ml)	Silver Acetate (µM)			
	OD <sub>600</sub>			
0	1	2	4	
0	3.54 ± 0.01	3.48 ± 0.02	3.50 ± 0.07	3.35 ± 0.10
4	1.71 ± 0.02	1.66 ± 0.09	0.95 ± 0.09	0.09 ± 0.07
8	0.68 ± 0.06	0.24 ± 0.02	0.14 ± 0.06	0.03 ± 0

Cultures of *E. coli* TOP10(pUC57PBLAAC2Ia) were performed in cation-adjusted Mueller Hinton with the indicated additions.

## 2.3. Bactericidal effect

Plazomicin showed bactericidal activity in previous studies [33]. Time-kill assays were used to evaluate if the phenotypic conversion to susceptibility observed when *E. coli* TOP10(pUC57AAC2Ia) was cultured in the presence of silver acetate in addition to plazomicin was due to a bactericidal effect. Figure 2 shows that adding silver acetate and plazomicin at a sub-MIC concentration had a robust bactericidal effect. Conversely, healthy growth was observed when one of the components was omitted. As in a previous report [33], regrowth was observed after 10 h incubation. It is worth

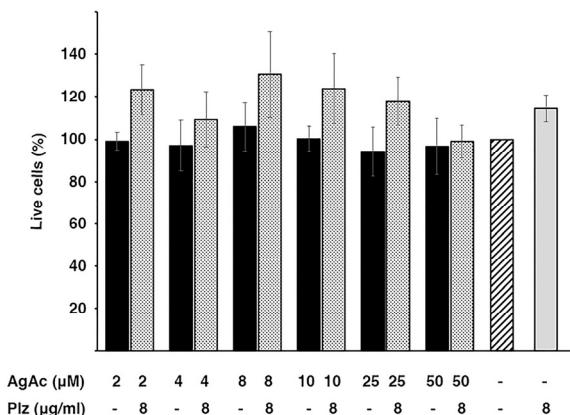
noting that in the past study, regrowth in time-kill assays was observed with concentrations of up to 2x or 4x MIC values depending on the strain assayed. The bases for regrowth remain to be elucidated. Possible causes are the emergence of resistance or presence of tolerant variants in the culture. The results of the experiments described in this section demonstrated that plazomicin, in the presence of  $\text{Ag}^{1+}$  ions, exerted bactericidal action on *E. coli* cells in which resistance is caused by the presence of the AAC(2')-Ia enzyme. The bactericidal effect of plazomicin plus  $\text{Ag}^{1+}$  ions on *E. coli* resistant cells was similar to that of plazomicin alone on susceptible *E. coli* cells.



**Figure 2.** Time-kill assays for plazomicin in the presence of silver acetate. *E. coli* TOP10(pUC57AAC2Ia) was incubated at 37 °C until the cell concentration reached the CFU/mL indicated in the figure at time zero. At this moment, the culture was divided in four aliquots and each one was supplemented with the compounds and concentrations indicated. The cultures were incubated at 37 °C, and CFU/mL values were measured at different intervals. AgAc, silver acetate. Assays were done in duplicate, and the values are mean  $\pm$  SD of two independent experiments.

### 2.3. Cytotoxicity of the mix plazomicin/silver acetate

An essential factor for the viability of combination therapies is that they show low toxicity to the host. A preliminary analysis of the cytotoxicity of the mix investigated in this work was carried out using HEK293 cells. Figure 3 shows that the exposure of the cells to the combination or the individual components at the active concentrations did not cause significant mortality. While these experiments are a preliminary step toward understanding the toxicity of the combination plazomicin/silver acetate, the results warrant further development towards overcoming the action of the AAC(2')-Ia enzyme.



**Figure 3.** Cytotoxicity of silver acetate and plazomacin. Cytotoxicity on HEK293 cells was assayed using a LIVE/DEAD kit as described in the Materials and Methods section. The percentage of surviving cells was calculated relative to cells untreated (striped bar). Control of maximum toxicity

was determined by incubating the cells in 70% methanol. Assays were done in triplicate and the values are mean  $\pm$  SD of three independent experiments.

### 3. Discussion

Infectious diseases are a leading cause of death, compromised health, and disability [34]. Outbreaks of bacterial infection, usually associated with multidrug resistance, are increasingly reported and may soon be responsible for millions of deaths per year [35-37]. Furthermore, the increase in hard-to-treat or untreatable bacteria also threatens medical procedures like surgery, cancer treatment and other chronic diseases, organ transplants, dental work, and care for premature infants [13, 38-40]. The impact of the drug resistance crisis is such that it was listed as one of the top ten global health threats [41]. Compounding the problem, unlike in the past when new antibiotics were available if existing ones became ineffective, the number of new antibiotics in development is dangerously low [1]. It is necessary to devise methodologies that extend the life of antibiotics currently in use. The development of inhibitors of mechanisms of resistance that can be administered in combination with the cognate antibiotic can be a viable strategy to treat resistant bacteria [11, 13]. Although there are no inhibitors of resistance to aminoglycosides in clinical use, this course of action has already been proven successful for  $\beta$ -lactamase-mediated resistance to  $\beta$ -lactams [42].

While plazomicin is a new aminoglycoside antibiotic, there are already enzymes that can inactivate it. AAC(2')-Ia catalyzes the inactivation of plazomicin by acetylation and reduces its potency [18]. Following the steps of previous work indicating that selected metal ions interfere with the acetylation reaction [26-29, 31, 43, 44], we tested the effect of  $\text{Ag}^{1+}$ . We chose this ion because, unlike other metals, ionophores were not necessary to observe reversion of resistance in growing bacterial cells when tested as an inhibitor of resistance to aminoglycosides mediated by AAC(6')-Ib [31]. Plazomicin was readily acetylated in vitro in a soluble extract of cells containing a recombinant clone harboring *aac(2')-Ia*. The AAC(2')-Ia activity results in resistance to plazomicin as determined by measuring MIC values for *E. coli* cells carrying recombinant clones that include the gene expressed under the control of the natural or the constitutive *bla<sub>TEM</sub>* promoters. Since both recombinant plasmids were generated using the same plasmid vector, the gene dosage in both strains must be identical. Therefore, the similarity of the MIC values showed by both strains suggests that the described transcriptional regulation of expression of *aac(2')-Ia* [45] does not impact resistance levels in the conditions used in our assays. The effect of silver acetate was also identical in the strains harboring the structural gene expressed under the control of both promoters. When plazomicin was present at 4 or 8  $\mu\text{g}/\text{ml}$ , the concentrations needed to overcome resistance were as low as 1 and 2  $\mu\text{M}$ , respectively. It is worth noting that it has been described that silver ions potentiate the effect of aminoglycoside and other antibiotics by mechanisms still in discussion. There is consensus that silver ions increase membrane permeability, which can enhance the effect of antibiotics against Gram-negative bacteria [46-48]. It has also been suggested that silver-mediated increased production of reactive oxygen species plays a role in potentiating the action of aminoglycosides [47]. However, this latter possibility has not been confirmed. Our prior [31] and present results indicate that silver ions also enhance the action of aminoglycosides by interfering with enzymatic inactivation, at least in the case of two aminoglycoside modifying enzymes (AAC(6')-Ib and AAC(2')-Ia). We conclude that silver ions potentiate aminoglycosides by multiple mechanisms that result in the observed phenotypic conversion to susceptibility. These facts, taken together with the confirmation that plazomicin retains its bactericidal action when acting in concert with  $\text{Ag}^{+1}$  to inhibit growth of resistant bacteria, and that the combination plazomicin/silver acetate at the active concentrations does not exhibit cytotoxicity, make these mixes excellent candidates to extend the useful life of plazomicin and other aminoglycosides.

#### 4. Materials and Methods

##### 4.1. Bacterial strains and plasmids

*Escherichia coli* TOP10 F- *mcrA*  $\Delta(mrr-hsdRMS-mcrBC)$   $\Phi80lacZ\Delta M15$   $\Delta lacX74$  *recA1* *araD139*  $\Delta(aral-leu)7697$  *galU* *galK* *rpsL(Str<sup>R</sup>)* *endA1* *nupG* was transformed with the plasmid pUC57AAC2Ia and used for all assays. The plasmid pUC57AAC2Ia was constructed inserting the *P. stuartii aac(2')*-*Ia* gene (accession number L06156, nucleotides 12-820)[45] into the *Bam*HI/*Hind*III sites of pUC57. The plasmid pUC57PBLAAC2Ia was generated fusing the *blatem* promoter and Shine-Dalgarno sequences, fragment encompassing nucleotides 4154-4225 (reverse complement, accession number J01749) [49] to the *aac(2')*-*Ia* open reading frame (fragment encompassed by nucleotides 264-810, accession number L06156) [45]. Transformation of *E. coli* TOP10 with pUC57AAC2Ia was carried out as described by Cohen et al [50].

##### 4.2. Bacterial growth

Bacteria were cultured in Lennox L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) with the addition of 2% agar in the case of solid medium. Plazomicin resistance levels were determined in cation-adjusted Mueller-Hinton broth at 37°C with shaking. Growth was assessed measuring the optical density at 600 nm (OD<sub>600</sub>) of the cultures containing the specified additions. Plazomicin was generously supplied by Cipla Therapeutics.

##### 4.3. MIC determination

MIC values were determined using plazomicin commercial E-strips (Liofilchem S.r.l., Roseto degli Abruzzi, Italy) following the recommendations of the supplier on Mueller-Hinton agar plates.

##### 4.4. Time-kill assays

Bacterial cells were cultured in cation-adjusted Mueller-Hinton broth until they reached 10<sup>7</sup> CFU/ml. At this time the cultures were divided in four aliquots, one of them was left intact and the others were supplemented with either 8 µg/ml plazomicin, 4 µM silver acetate, or both. Incubation was continued at 37°C with shaking and the CFUs were determined after the indicated times [29].

##### 4.5. Acetyltransferase assays

Total soluble proteins (enzymatic extracts) were prepared as before [31]. Briefly, cells were recovered from cultures by centrifugation, resuspended in a 0.5 mM MgCl<sub>2</sub> solution, and sonicated with a Heat Systems Ultra- sonic, Inc., Model No. H-IA (Plainview, NY) cell disrupter. The soluble proteins were then separated by centrifugation in a microfuge for 10 min at 4°C. The protein content of the extracts was determined using a commercial reagent (Bio-Rad). Acetyltransferase activity was assessed using the phosphocellulose paper binding assay [51]. Soluble extract (120 µg protein) obtained from *E. coli* TOP10(pUC57AAC2Ia) cells was added to the reaction mixture (200 mM Tris HCl pH 7.6 buffer, 0.25 mM MgCl<sub>2</sub>, 330 µM plazomicin, the indicated concentrations of sodium acetate or silver acetate, and 0.05 µCi of [acetyl-1-<sup>14</sup>C]-acetyl-coenzyme A (specific activity 60 µCi/µmol) in a final volume of 30 µl. After incubating the reaction mixture at 37 °C for 30 minutes, 20 µl were spotted on phosphocellulose paper strips. The unreacted radioactive substrate was washed once by submersion in 80 °C water followed by two washes with room temperature water. The phosphocellulose paper strips were then dried and the radioactivity corresponding to acetylated plazomicin was determined.

##### 4.6. Cytotoxicity assays

The cytotoxicity of the combination silver acetate/plazomicin on HEK293 cells [52] was assessed as previously described [53]. One thousand cells per well were cultured on flat-bottom 96-well, black microtiter plates for 12 h before addition of the compounds to be tested. Incubation was then

continued for 24 h before the cells were washed with sterile D-PBS, resuspended in the LIVE/DEAD reagent (2  $\mu$ M ethidium homodimer 1 and 1  $\mu$ M calcein-AM) (Molecular Probes), and incubated for 30 min at 37°C. At this moment the fluorescence levels at 645 nm (dead cells) and 530 nm (live cells) were measured. The percentage of dead cells was calculated relative to the untreated control cells. Control of maximum toxicity was calculated on cells treated with 70% methanol for 20 min. Experiments were conducted in triplicate. The results were expressed as mean  $\pm$  SD of three independent experiments.

## 5. Conclusions

There is an urgent need for novel strategies to extend the useful life of antibiotics currently in use. Latest-generation antibiotics such as plazomicin can quickly be overcome by resistance mechanisms developed by bacteria. The ability of Ag<sup>1+</sup> to interfere with the action of AAC(2')-Ia, an aminoglycoside modifying enzyme that mediates the inactivation of plazomicin, makes it an excellent candidate as a plazomicin adjuvant to eliminate this enzyme as a threat to the effectiveness to this antibiotic. The low cytotoxicity observed at the active concentrations makes the combination plazomicin/Ag<sup>1+</sup> a viable option for treating multidrug resistant infections.

**Author Contributions:** Conceptualization, D.N. and M.E.T.; methodology, D.N., A.M., T.T., K.P., V.J., M.S.R., and M.E.T.; formal analysis, D.N., A.M., T.T., V.J., M.S.R., and M.E.T.; investigation, D.N., A.M., T.T., J.S., V.J., M.S.R., and M.E.T.; resources; writing—original draft preparation, M.E.T.; writing—review and editing, D.N., A.M., J.S., B. E., K. P., V.J., M.S.R., and M.E.T.; supervision, M.E.T.; funding acquisition, M.E.T.; project administration, M.E.T. All authors have read and agreed to the published version of the manuscript.

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