POTENTIAL GENOTOXICITY OF ACETAMIPRID AND PROPINEB SINGLY OR IN COMBINATION IN CULTURED HUMAN PERIPHERAL BLOOD LYMPHOCYTES BY USING MN ASSAY

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ABSTRACT

Acetamiprid, a neonicotinoid insecticide, and Propineb, a dithiocarbamate fungicide, are used in agriculture singly or in combination with other pesticides. The aim of the study is to investigate the single and combined genotoxic effect two pesticides on cultured human lymphocytes by using the Micronucleus (MN) assay. The lymphocytes were treated with three different concentrations of pesticides singly and in combination (a mix), Acetamiprid (0.625, 1.25, 2.5 µg mL⁻¹), Propineb (12.5, 25, 50 µg mL⁻¹) and Acetamiprid+Propineb (0.625+12.5, 1.25+25, 2.5+50 µg mL⁻¹), for 24 and 48 hours. MN frequency significantly increased at a 48-hour exposure period when the lymphocytes were exposed to the combined pesticides. The data demonstrated that these pesticides may have a synergistic effect and may have potential genotoxic activity in cultured human lymphocytes. Also, it was shown that when they are combined and exposed to lymphocytes these pesticides have a cytotoxic effect.

KEYWORDS:
Acetamiprid; Propineb; in vitro; human lymphocyte culture

1. INTRODUCTION

Different classes of pesticides are used singly or in combination against living organisms in agricultural areas to increase crop yield. Because pesticides are so important to agriculture, it is important to evaluate if they are harmful to humans.

Compounds in mixtures may interact with one another and with biological systems. They may also alter the genotoxicity of individual compounds [1]. Occupational or environmental exposure to pesticide mixtures was associated with increased genotoxicity in human lymphocytes [2-4].

Although there are many studies on the genotoxicity of pesticides, genotoxicity studies of the effects of mixing different forms of pesticides are limited. It is important to study pesticide formulations singly and in combination. Pesticides may have greater genotoxic effects in combination form than when they are used singly. The exposure of non-toxic doses of atrazine and alachlor when combined induced significant frequencies of chromatid breaks and fragments in mouse bone marrow cells; individual exposure, however, in similar dosages did not induce any significant effect [5]. Dolara et al. [6] and Roloff et al. [7] also demonstrated that the effect of combined pesticides is much greater than that of an individual pesticide.

Acetamiprid (ACET) is a neonicotinoid group pesticide used against insects both in the agricultural and the domestic areas [8]. These systemic insecticides are agonists of nicotinic acetylcholine receptors (nAChR) [9]. Neonicotinoid insecticides have significant toxicity to insects but have low toxicity to mammals because of the stronger affinity of neonicotinoids to insects’ nAChR than to mammalian nAChR [10, 11]. In in vivo and in vitro studies, the Environmental Protection Agency (EPA) reported that Acetamiprid is neither mutagenic nor genotoxic [12]. But some studies have revealed genotoxic and cytotoxic effects of neonicotinoid insecticides in culture of human lymphocytes [13-15]. It was demonstrated that the 25-40 µg mL⁻¹ commercial form of Acetamiprid has genotoxic and cytotoxic potential on human peripheral lymphocytes in vitro [13]. Calderon-Segura et al. (2012) reported genotoxic and cytotoxic effects on peripheral blood lymphocytes following in vitro exposure to a commercial formulation of four neonicotinoid insecticides (Calypso, thiacloprid; Poncho, clothianidin; Gaucho, imidacloprid and Jade, imidacloprid) by using a comet assay [16]. A significant increase in MN frequency was reported in human peripheral lymphocytes with both the pure and the commercial formulations of a neonicotinoid insecticide (imidacloprid) [17].

Propineb (PROP) is a dithiocarbamate, a class of compounds with high biological activity, extensively used as a fungicide in agriculture [18]. Propineb is bio-transformed into propylene-thiourea (PLTU) and carbon disulfide (CS₂)
and release zinc (Zn$^{2+}$) [19]. Rolandi et al. [20] stated that Propineb and its main metabolite propylene-thiourea did not increase MN frequency in mice bone marrow. Propineb gave negative results in the following studies: the Ames test of S. typhimurium [21, 22], the HGPRT test in Chinese hamster ovary (CHO) cells [23], an unscheduled DNA synthesis test (UDS) in rat hepatocytes [24] and an MNi in mouse bone marrow [25]. Some studies have revealed the genotoxic and cytotoxic effects of dithiocarbamate fungicides on animal tissues [20, 26-30]. Despite a low acute toxicity [31], dithiocarbamates have shown teratogenic, goitrogenic and neurotoxic activity in animal studies [31, 32]. Propineb has low acute toxicity with a specific goitrogenic effect on rats [27]. There are no data that reveal its genotoxicity profile in humans.

Commercial formulations are the commonly used form of pesticide in agriculture; as a result of this use, people, in particular, agricultural workers, may be exposed to pesticide formulation combinations. An investigation is necessary to determine genotoxic effects. The MN assay is a useful method for evaluating the genotoxic effect of chemicals and assessing DNA damage at the chromosomal level. MN is one of the cytogenetic markers used to detect early biological effects related to DNA-damaging compounds [33]. The cytokinesis-block MN (CBMN) assay has been widely used on different cell types, including human lymphocytes, to evaluate the clastogenic and aneugenic potential of various agents. MN can be formed from acentric fragments or from the failure of the mitotic apparatus [34, 35]. An increased MN frequency in peripheral blood lymphocytes implies a cancer risk in humans in vivo [35, 36].

The present study aimed to investigate the single and combined genotoxic effect of two pesticide formulations Mosetam 20SP® (20% active ingredient); and Antracol 70WP® (containing 70% active ingredient) in cultured human lymphocytes using the MN assay.

### 2. MATERIALS AND METHODS

#### 2.1 Study design

In this study, human peripheral blood was used as target cells and the commercial formulations of Acetamiprid (Mosetam 20SP; containing 20% active ingredient; obtained from Safa Agriculture) and Propineb (Antracol 70WP; containing 70% active ingredient; obtained from Bayer) were used as test substances. The concentrations were selected according to preliminary studies that the lymphocytes were exposed to Acetamiprid (0.625, 1.25, 2.5 $\mu$g mL$^{-1}$), Propineb (12.5, 25, 50 $\mu$g mL$^{-1}$) and Acetamiprid+Propineb (0.625+12.5, 1.25+25, 2.5+50 $\mu$g mL$^{-1}$). Whole blood samples from 4 volunteers (2 female and 2 male), who were healthy, between 20 and 25 years of age, non-smoking and with no history of pesticide exposure, were used in cultures for MN analysis. For each culture, 0.3 mL of whole blood was added to 4.7 mL Ham’s F-10 (Sigma) medium supplemented with 20% fetal bovine serum, 2% phytohemagglutinin M (PHA) and antibiotics (100 IU penicillin mL$^{-1}$ and 100 µg streptomycin mL$^{-1}$). The blood was cultured at 37°C for 70 hours. Cytochalasin B (Cyt-B, CAS no 14930-96-2) (final concentration 6 µg mL$^{-1}$) was added to the cultures 44 hours after the initiation of the cultures. Test chemicals were added at the 46th and 22nd hours of the culture period for 24 hours and 48 hours of exposure respectively. Also, 0.2 µg mL$^{-1}$ Mitomycin C (MMC) was used as a positive control and distilled water as a negative (solvent) control. The cytokinesis block micronucleus assay (CBMN) was done using the Standart technique proposed by Fenech in 2007 [37]. The number of MN in 2,000 binucleated (BN) cells per donor (a total of 8,000 binucleated cells per each concentration and exposure period) was scored. A total of 2,000 cells (500 cells per donor for each concentration and exposure period) were scored for the determination of the Nuclear Division Index (NDI). The NDI was calculated as follows: NDI= (M1+2.M2+3.M3+4.M4)/n. M1 to M4 indicates the number of nuclei (cells from 1 to 4), and n stands for the total number of cells recorded. All donors were informed about the study and they willingly signed using blood samples in the study. The investigation was performed with ethical standards according to the Declaration of Helsinki.

#### 2.2 Statistics

The statistical analysis was done according to the total data from 4 donors. Fisher’s exact X$^2$ test was used to evaluate the significance of the difference between the exposure and control groups of MNi frequencies. The X$^2$ test was used for NDI values. The level of P<0.05 was considered statistically significant. Evaluating mixed-exposure data with single-exposure data, we compared our measured values with the expected values. The expected value was calculated as a mean value obtained after exposure to one substance alone plus a mean value obtained after exposure to a second substance [38, 39], e.g.:

$$\text{Mean}_{\text{expected}}(\%) = \text{mean}_{\text{substance1}}(\%) + \text{mean}_{\text{substance2}}(\%) - 100(\%)$$

The expected SEM (Standart Error Mean) was calculated as follows:

$$\text{SEM}_{\text{expected}} = \sqrt{\left(\text{SEM}_{\text{substance1}}\right)^2 + \left(\text{SEM}_{\text{substance2}}\right)^2}$$

The difference was calculated between the expected and measured values using an unpaired t test (level of P<0.05). The results were interpreted as follows: an additive effect was implied if the measured values were not significantly above or below the expected values; a synergistic effect was implied if the measured values were significantly above the expected values; an antagonistic effect was implied if the measured values were significantly below the expected values [39].

### 3. RESULTS

Three concentrations of each pesticide (Acetamiprid: 0.625, 1.25, 2.5 $\mu$g mL$^{-1}$; and Propineb: 12.5, 25, 50 $\mu$g mL$^{-1}$; and...
mL⁻¹) and their mixed forms (Acetamiprid+Propineb: 0.625+12.5, 1.25+25 and 2.5+50 µg mL⁻¹) were tested for 24- and 48-hour exposure periods in the human peripheral blood lymphocyte culture. The total results of four donors are shown in Tables 1 and 2 related to exposure periods 24 and 48 h. Although there are some individual differences between donors (not shown in the tables), the total values of the four donors showed that ACET and PROP did not significantly increase MN frequency, except for 12.5 µg mL⁻¹ PROP for the 24-hour exposure period. It was observed that the mixed form of these pesticides significantly increased MN frequency in the 48-hour exposure period (p<0.01 and p<0.001) (Table 2). The measured values were significantly above the expected values which may indicate a synergistic effect [38, 39]. The BNMN% values of ACET, PROP and MIX exposures for the 24 and 48-hour exposure periods are shown in Figure 1. The results showed that an increase in MN frequencies was higher than that of the additive effect, which may means a synergistic effect. In Figure 2, a dose-related increase was demonstrated in MN frequencies after exposure to a mixture of insecticides after 24 and 48-hour periods. Also, in the present study, the use of combined pesticides decreased the NDI over a 48-hour exposure period. According to the results, MN frequency increased with a decrease in NDI values. But these pesticides showed no cytotoxic effect in single (24 hours and 48 hours) or combined (24 hours) exposures, except 1.25 µg mL⁻¹ at 48 hours Acetamiprid exposure.

TABLE 1 - Frequency of micronuclei and nuclear division index in human lymphocytes exposed to ACET, PROP and ACET+PROP for 24 hour exposure period (Total values of 4 donors).

<table>
<thead>
<tr>
<th>Exposure period 24 hours</th>
<th>Concent. µg/ml</th>
<th>BN cells according to No.MNi</th>
<th>MN</th>
<th>BNMN ±S.E.</th>
<th>BNMN %net.S.E.</th>
<th>NDI ±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(-) Control</td>
<td>7953</td>
<td>41</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>MMC</td>
<td>7707</td>
<td>269</td>
<td>18</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>ACET</td>
<td>0.625</td>
<td>7956</td>
<td>30</td>
<td>12</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>7934</td>
<td>52</td>
<td>12</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>7914</td>
<td>64</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PROP</td>
<td>12.5</td>
<td>7878</td>
<td>114</td>
<td>6</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>7894</td>
<td>100</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>7892</td>
<td>102</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>ACET+PROP (MIX)</td>
<td>0.625+12.5</td>
<td>7931</td>
<td>61</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.25+25</td>
<td>7918</td>
<td>67</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.5+50</td>
<td>7908</td>
<td>89</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Concent, Concentration; MN, total N; BNMN, binucleated cells with MN; NDI, nuclear division index. BNMN%±S.E., Mean number of MN of 1000 binucleated cells±Standard Error; the significant differences among treated groups by analysis of X² test to *p<0.05; **p<0.01; ***p<0.001

TABLE 2 - Frequency of micronuclei and nuclear division index in human lymphocytes exposed to ACET, PROP and ACET+PROP for 48 hour exposure period (Total values of 4 donors).

<table>
<thead>
<tr>
<th>Exposure period 48 hours</th>
<th>Concent. µg/ml</th>
<th>BN cells according to No.MNi</th>
<th>MN</th>
<th>BNMN ±S.E.</th>
<th>BNMN %net.S.E.</th>
<th>NDI ±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(-) Control</td>
<td>7967</td>
<td>27</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>MMC</td>
<td>7588</td>
<td>356</td>
<td>42</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>ACET</td>
<td>0.625</td>
<td>7968</td>
<td>24</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>7954</td>
<td>34</td>
<td>8</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>7940</td>
<td>42</td>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PROP</td>
<td>12.5</td>
<td>7966</td>
<td>24</td>
<td>6</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>7936</td>
<td>50</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>7940</td>
<td>48</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ACET+PROP (MIX)</td>
<td>0.625+12.5</td>
<td>7851</td>
<td>127</td>
<td>20</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.25+25</td>
<td>7829</td>
<td>135</td>
<td>34</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.5+50</td>
<td>7819</td>
<td>143</td>
<td>33</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

Concent, Concentration; MN, total N; BNMN, binucleated cells with MN; NDI, nuclear division index. BNMN%±S.E., Mean number of MN of 1000 binucleated cells±Standard Error; the significant differences among treated groups by analysis of X² test to *p<0.05; **p<0.01; ***p<0.001
A

FIGURE 1 - The values of BNMN ‰ were shown after 24 h (A) and 48 h (B) exposure of ACET, PROP and MIX. Dose 1, 2 and 3 represent first, second and third concentrations of each pesticide and their mixed concentrations. *p<0.05; **p<0.01; ***p<0.001

B

FIGURE 2 - Dose related increase of MN frequencies after exposure of mixture of pesticides at 24 and 48 h period.

4. DISCUSSION

In this study, two pesticides Acetamiprid (Mosetam 20SP; containing 20% active ingredient) and Propineb (Antracol 70WP; containing 70% active ingredient) were studied in singly or in combination exposures on human peripheral blood lymphocyte culture by using MN assay. The results demonstrated that ACET (at 0.625, 1.25, 2.5 µg mL⁻¹)
Acetamiprid (Mosetam 20 SP) at 25, 30, 35 and 40 µg mL\(^{-1}\) concentrations did not increase MN frequency significantly when used individually. But when they were used in a mixture, the MN frequency increased significantly at 48 h and a reduction in MN was observed at 24 h.

In this study, ACET was not genotoxic and cytotoxic at tested concentrations. The other studies demonstrated that ACET was genotoxic at cytotoxic levels. In the study of Kocaman and Topaktaş [13], the commercial form of Acetamiprid (Mosetam 20 SP) at 25, 30, 35 and 40 µg mL\(^{-1}\) concentrations was used in human peripheral blood lymphocytes to evaluate the genotoxic effect. The researchers stated that except for the lower concentration (25 µg mL\(^{-1}\)), the other concentrations induced MNi formation. Çavaş et al. (2012) stated that Acetamiprid induced MNi at cytotoxic levels for CaCo-2 cells [15]. The researchers demonstrated that the 25 µM Acetamiprid concentration is not cytotoxic and genotoxic, although the concentrations between 50 µM and 300 µM have genotoxic and cytotoxic potential [15]. Similarly, in this study, much lower concentrations were used and did not significantly induce MNi formation. The results support each other in finding that lower concentrations do not induce MNi in human peripheral blood lymphocytes. The EPA [12] also stated that ACET was not genotoxic at cytotoxic levels. In the study of Roloff et al. (1992), the same result was stated that an independent exposure period of non-toxic doses of atrazine and alachlor increased significant frequencies of chromatid breaks and fragments in mouse bone marrow cells, whereas the combined exposure of non-toxic doses of atrazine and alachlor increased significant frequencies of chromatid breaks and fragments in mouse bone marrow cells, whereas the independent exposure to similar doses failed to induce any significant effect [5]. Densia et al. (2007) do not indicate a clear-cut genotoxic effect of imidacloprid (chloronicotinyl insecticide) and metalaxyl (benzenoid fungicide) when tested separately on human lymphocytes in vitro or in vivo.

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on rat bone marrow in vivo. But in treatments with a mixture of these pesticides, they observed a statistically significant difference at the tested concentrations for both SCE and MNPCEs[4]. Pesticide mixtures show an increase in the frequency of chromosomal aberrations, DNA adducts formation and reactive oxygen species that can disrupt genetic integrity and alter the biochemistry of metabolic pathways [49, 50].

In this study, statistical results indicated that the combined effect of two chemicals is greater than the sum of the effects of each chemical given alone, which indicates their synergistic effect. Similarly, in the study of Chauhan et al. (2005), it was demonstrated that the commercial formulations of a cypermethrin and quinalphos insecticides mixture have synergistic genotoxicity in mice [51]. Rencizoğulları and Topaktaş (1998) reported a synergistic effect between Carbosulfan-Ethyl carbamate and Carbosulfan-ethyl-methanesulfonate, which increased the formation of SCE in human peripheral lymphocytes [52]. The synergistic effect of methamidophos and imidacloprid causes an increase in potential damage to on-target organisms [53]. Das et al. (2007) reported that binary mixtures of monocrotophos, carbofuran and endosulfan have a synergistic effect on human peripheral blood lymphocytes [3]. High concentrations of individual pesticides cause significant DNA damage, and lower concentrations of their binary mixtures could cause the same effect. Đikic et al. (2012) reported a synergistic effect between the pesticides imazalil (imidazole fungicide), cypermethrin (pyrethroid insecticide) and carbendazim (carbamate fungicide) in in vivo experiments by using a comet assay. Carbendazim combined with imazalil or cypermethrin create potential DNA damage in hepatocytes of mice[54]. In most of the investigations, it was reported that although very low concentrations of non-toxic, non-genotoxic pesticides were used, they had a genotoxic effect on living organisms when exposed in mixed forms.

This synergistic effect may cause by the formation of more toxic products through chemical interactions between the two parent compounds in the mixture. Synergism is observed when the effect of exposure to a pesticide mixture is much greater than the one expected from an additive effect, and responses cannot predicted by the known toxicology of each compound [55]. Certain insecticide combinations show a clear pattern of synergism at low, environmentally relevant concentrations [56, 57]. Not all mixtures of pesticides with similar chemical structures always produce additive effects; thus, different toxic effects can be observed if the pesticides act on multiple sites. Despite the similarities in their chemical structure, pyrethroids (PYRs) act on multiple sites and their mixtures produce different toxic effects [55]. Exposure to mixtures of different classes of pesticides produces effects that are often difficult to anticipate. Permethrin and the carbamate propoxur elicited greater-than-additive toxicity because of the complementary modes of the toxic action of PYR and N-methylcarbamates NMC, two insecticide classes that act on different components of nerve impulse transmission. Diazinon (an organophosphate (OP)) and benomyl (a benzimidazole fungicide), which individually fail to exhibit genotoxicity, are genotoxic when administered together [55]. DNA damage could be monitored at higher concentrations of pesticides when these pesticides are exposed individually; the same genotoxic effect could also be obtained at very low concentrations of pesticide mixtures.

Metabolic interactions of pesticides involve the inhibition or induction of detoxifying enzymes, and can occur not only between two or more of these compounds but also between pesticides and endogenous substrates. Potentially may occur when one of the components of the mixture changes the toxicokinetics of the pesticide as a result of its increased activation or decreased detoxification, leading to enhanced or reduced toxicity, respectively[58]. The synergism between the pesticide mixture and adjuvants used as emulsifiers, stabilisers and solvents cannot be ruled out. However, it cannot be ascertained unless products of formulation are tested separately with the individual pesticide or the pesticide mixture. The tissue doses of chemicals in a mixture can be predicted by using physiologically based toxicokinetics (PBTK) models. This requires information about the interaction of all the components in the mixture. And this information has to be determined by experimentation.

In this study, NDI values were not decreased after ACET exposure, except 1.25 µM concentration at a 48-hour exposure period, because of the low concentrations of the pesticides. NDI values were significantly decreased after 48-hour PROP and ACET+PROP exposures. PROP, polymeric zinc 1,2-propylenebis (DTC-dithiocarbamate), contains Zn²⁺ which can be released following its bio-transformation in the biological milieu [19]. The intact DTC molecules exhibit both pro-oxidant and antioxidant activities[59-64]. Whereas the disulfide bridges and the metal complexes contribute to their pro-oxidant effects, the SH contributes to their antioxidant effects[61, 65]. The tissue organ-specific toxic effects of these chemicals may be caused by crucial structural and functional entities of the cells, eventually leading to metabolic disruptions, pathological changes and cell death [66].

The results show that low non-toxic concentrations of pesticides substantially increase genotoxicity when used in mixed forms. Although these pesticides have different toxicity mechanisms individually, the synergistic effect observed in this study might be caused by their combined mechanisms that increase effectiveness.

4. CONCLUSION

This study reveals the genotoxic effect of the single and combined exposure of Acetamipрид and Propineb pesticides on human peripheral lymphocytes by using an MN assay. Although at lower concentrations Acetamiprid and
Propineb are not genotoxic and cytotoxic individually, it was observed that the exposure to these pesticides when combined may have synergistic effect at 48-hour exposure periods on lymphocytes and a significant increase in MNI frequency; and a significant decrease in NDI values occurred. More genotoxic studies with different biological test systems are necessary to investigate that the use of Acetamiprid and Propineb when combined may be hazardous to animals, including humans. Further studies regarding their kinetics, dosimetry, dose-response and interaction with each other and the tissue, which will benefit synergistic risk assessment, are required to determine safe limits for humans and the environment.

ACKNOWLEDGEMENTS

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The authors have declared no conflict of interest.

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