Full Length Research Paper

Possible antiviral activity of montelukast against Herpes Simplex Virus type-1 and Human Adeno Virus *in vitro*

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Montelukast (MLS) is used for the treatment of various allergic reactions. Viral infections are important, especially in exacerbation of allergic diseases. In this study, the apoptotic/necrotic responses of Herpes Simplex Virus (HSV-1 and Human Adenovirus (HAdV) isolate in HEp-2 and Vero cell cultures were compared with the presence or absence of non-toxic doses of pure MLS. The non-toxic dose of pure MLS for both cell lines was found to be 9.76 µg/ml. The apoptotic cell (APC) ratios of HEp-2 cells infected with HSV and HAdV were 12.5 and 9.1% before MLS and 10.3 and 7.7% after MLS, respectively. The necrotic cell (NEC) ratio of HEp-2 cells infected with HSV and HAdV were found 26 and 20% before MLS and were found to be 22.0 and 18.7% respectively after MLS. APC ratio of Vero cells infected with HSV and HAdV were found 16 and 12.85% before MLS and were found to be 14.6 and 10.4% respectively after MLS. NEC ratio of Vero cells infected with HSV and HAdV were found to be 34 and 28.2% before MLS and were found to be 30 and 26.4% respectively after MLS. The tissue culture infectious doses ($TCID_{50}$) values of HSV-1 for HEp-2 cells and Vero cells without MLS were $10^{-5,25}$/0.1 ml and $10^{-4,95}$/0.1 ml, with MLS were $10^{-4,15}$/0.1 and $10^{-3,75}$/0.1 ml, respectively. The $TCID_{50}$ values of HAdV for HEp-2 cells and Vero cells without MLS were $10^{-7,0}$/0.1 and $10^{-6,75}$/0.1 ml, with MLS were $10^{-6,25}$/0.1 and $10^{-5,75}$/0.1 ml, respectively. Although the methods applied in this study provides opportunities for morphologic diagnosis; results suggest the possible antiviral effect of montelukast.

Key words: Montelukast, simplexvirus, adenoviridae, atopy, Vero cells, HEp-2 agents.

INTRODUCTION

Cysteinyl leukotrienes (CysLTs) are lipoxygenase products derived from the metabolism of arachidonic acid and they are potent endogenous mediators of inflammation. Montelukast is an leukotriene receptor antagonist (LTRA) that acts as an antagonist of CysLT1R, blocking its signal transduction without affecting cysteinyl-LT signalling through CysLT2R, or the action of leukotriene B4 (LTB4) via the BLT receptors.

The LTRA agents are well tolerated and an effective medication for the treatment of asthma. Furthermore, as an anti-inflammatory drug, recent studies have demonstrated beneficial effects of LTRAs on different allergic diseases (asthma, rhinitis, chronic urticaria, atopic dermatitis, allergic fungal disease), as well as other diseases not connected to allergic situations (Riccioni et al., 2004).

Leukotrienes (LTs) are synthesized in response to different immune and inflammatory stimuli, and viruses are also important in triggering such an inflammatory response. Besides this, viruses are extremely potent infectious agents and there is no doubt that, in certain clinical situations, infections with some viral agents, for example adenovirus and herpes simplex virus, can be associated with allergic diseases.

Human adenoviruses (HAdV) are estimated to cause~5

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to 10% of childhood respiratory infections (Chuang et al., 2003); symptoms of respiratory illness caused by adenovirus infection range from the common cold syndrome to pneumonia, croup and bronchitis. HAdV has invariably been linked with asthma exacerbation (Tan, 2005). Some reports suggest that persistent adenovirus infection serves as a risk factor for childhood asthma (Macek et al., 1994; Marin et al., 2000).

Patients affected by atopic dermatitis (AD) tend to develop viral infections. The most commonly recognized viral complication in subjects with AD is eczema herpeticum (EH), which is caused by an extensive cutaneous infection, with herpes simplex viruses (HSV) (Beck et al., 2009).

Some studies have suggested that LTRA agents may also have antiviral effects. Bisgaard et al. (2005) suggested that montelukast effectively reduced asthma exacerbations in 2 to 5 years old patients. And because viral infections are the major reason for asthma exacerbation, especially in this age group, these authors also suggested that a possible explanation for such a reduction could be the antiviral effect of montelukast (Bisgaard 2003). In our previous study, we also found that there might be a protective effect of MLS against viral infections that could lead to the exacerbation of asthma (Igde and Anlar, 2009).

Naturally, leukotrienes appear to play a critical role in the pathogenesis of inflammation of both allergic diseases and viral infection. It is known that viral infections are important in the natural course of allergic diseases, and some data also support MLS as having anti-inflammatory rather than anti-allergic properties, which may signal its antiviral effect. Despite all this, the relationship between MLS and viral infection has not yet been elucidated.

In our study, by observing the apoptotic/necrotic cell response of cultured tissue cells to infective doses of herpes simplex virus and human adenovirus with and without MLS at a non-toxic dose, we attempted to evaluate the antiviral effect of MLS.

MATERIALS AND METHODS

Cell lines and viruses:

Human laryngeal epithelial carcinoma (HEp-2) and African Green Monkey Kidney (Vero) cell lines were used in this study. The HEp-2 cell line was grown in Eagle's minimum essential medium (EMEM, Biological Industries, Israel) with 5% foetal calf serum (FCS, Biological Industries, Israel) and 1% penicillin/streptomycin (Biochrome, KG). The Vero cell line was grown in Dulbecco's minimum essential medium (DMEM, Biological Industries, Israel) with 10% foetal calf serum (FCS, Biological Industries, Israel) and 1% penicillin/streptomycin (Biochrome, KG). The cells were maintained in a 5% CO_2 atmosphere with 95% humidity at 37°C.

The KOS strain of HSV-1 and human adenovirus isolate were used in this study (Baskin et al., 2005; Yazici et al., 2005). When the HEp-2 and Vero cell monolayers reached 90% confluency they were infected with the KOS strain of HSV-1 and the HAdV isolate in 75 cm^2 cell-culture flasks and incubated at 37°C in a humidified

incubator with 5% CO_2 for between 2 to 4 days until cytopathic effects (CPE) were observed. Following a single freeze-thaw cycle, 1 ml aliquots of viral infected HEp-2 and Vero lysates were stored at -80°C until further analyses.

Assay to determine the infectivity of the viruses

The infectivity of the viruses was determined using the microtitration technique on HEp-2 and HAdV cells according to the method of Frey and Liess (1971). Briefly, serial tenfold (10^{-1} to 10^{-8}) dilutions of each virus in MEM were prepared. An aliquot (50 µl) of each dilution was added to each of four wells/dilutions in a 96-well tissue culture plate. A 50 µl suspension of HEp-2 (550,000 cells/ml) was added to each well containing HSV-1 and HAdV. The same procedure was repeated with Vero cells (700,000 cells/ml).

Calculation of non-toxic doses of montelukast sodium (MLS)

The non-toxic doses of MLS were separately calculated for HEp-2 and Vero cells using the microtitration plate technique (Baskin et al., 2005). Briefly, serial two-fold dilutions of MLS in MEM were prepared: from 10,000 to 0.61035 µg/ml. Equal amounts of serially diluted MLS were dispensed (100 µl) into three wells in 96-well tissue culture plates (Greiner, Germany) for each concentration. Subsequently, 100 µl of homogeneous HEp-2 cell suspensions (550,000 cells/ml) were added to each MLS dilution. The same procedure was performed for the Vero cells (700,000 cells/ml).The plates were then incubated in a humidified, 5% CO_2 atmosphere, at 37°C, for three days. The cells were monitored by daily microscopic examinations. The non-toxic dose of MLS was determined by staining the cells with the vital dye trypan blue. At toxic doses no blue colour was observed in the cells because of cell death, but at non-toxic doses the cells were coloured blue. These experiments were repeated three times.

Morphological assessment of apoptosis and cell death

For the determination of apoptosis and cell death, the vital dyes propidium iodide (Sigma) and Hoechst 33342 (bisbenzimide H 33342, Sigma) were used. This method relies on differences in the permeability of cell membranes of live, dead and apoptotic cells and is time dependent (Baskin et al., 2005). Briefly, the cells were fixed with 1 to 3% formaldehyde in PBS at RT for 20 min, the fixing solution was aspirated, cold 96% ethanol added, and the cells were left at room temperature for 20 min. The ethanol was then aspirated, rinsed three times with PBS and the cells were incubated for 15 min at 37°C with Hoechst 33342 dye (5 µg/ml in PBS), before being rinsed three times with PBS. Propidium iodide (50 µg/ml from a 1 mg/ml stock in PBS) was added just before microscopy. The cells were monitored using a fluorescent microscope at a wavelength of 330 and 420 nm. A minimum of 200 cells were counted in in five different areas of the each well. This experiment was repeated three times and the cells were classified according to three criteria as follows (Baskin et al., 2005):

Live cells

Normal nuclei, pale blue/green chromatin with an organized structure.

Apoptotic cells

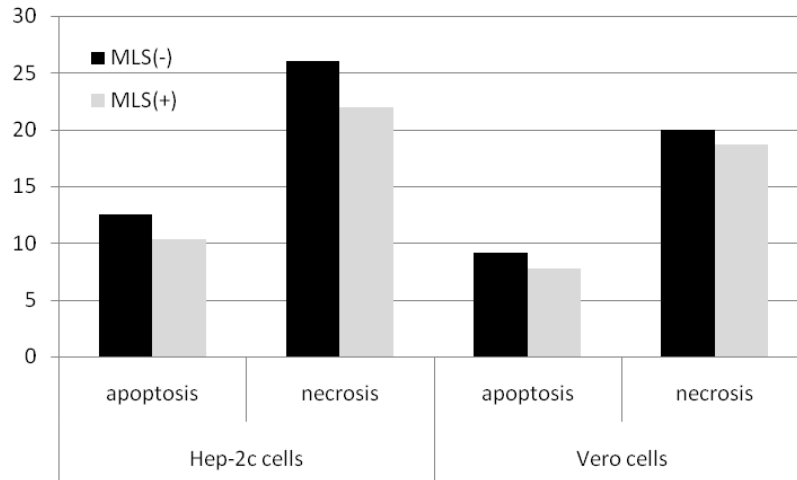Identified by chromatin condensation within the nucleus, intact

Figure 1. Apopitotic/necrotic behaviors of cells infected with HSV-1 with/without montelukast.

nuclear boundaries, bright blue chromatin and nuclear fragmentation into smaller nuclear bodies within an intact cytoplasmic membrane.

Necrotic cells

Identified by damage to the cytoplasmic membrane with a fairly intact nucleus, enlarged red nuclei, loss of the cytoplasm, damaged/ irregular nuclear membranes and slightly condensed nuclei that are dyed a bright red colour.

Statistical analysis

All statistical analyses were performed using SPSS Windows version 16.0 (SPSS Inc., Chicago, IL, USA). The chi square test was used to compare percentages and p values of less than 0.05 were considered statistically significant.

RESULTS

The non-toxic dose of montelukast was found to be 9.76 µg/ml. In the absence of HSV or HAdV infection, using serial dilutions of montelukast (10 to 0.00061 mg/ml), the apoptotic behaviours of HEp-2 and Vero cells were examined after 24 h of incubation at 37°C and 5% CO_2. Following this period, HEp-2 and Vero cells were examined by Hoechst 33342 (HOE)-propidium iodide staining for apoptosis/necrosis determinations. At a dose of 9.76 µg/ml, and at more diluted doses, montelukast showed no toxic or apoptotic/necrotic effects on HEp-2 or Vero cells.

Apoptotic cell (APC) ratio of HEp-2 and Vero cells infected with HSV-1 were lower in montelukast diluted cells (p<0.05). APC and necrosis ratios of HEp-2 and Vero cells infected with HSV-1 were found 12.5 and 9.1% before MLS administration and were found 10.3 and

7.7% respectively after MLS administration. Necrotic cell (NEC) ratio of HEp-2 and Vero cells infected with HSV-1 were found 26 and 20% before MLS administration and were found 22 and 18.7% respectively after MLS administration (Figure 1).

APC and NEC ratios of HEp-2 and Vero cells infected with HAdV were lower in montelukast diluted cells (p<0.05). APC ratio of HEp-2 and Vero cells infected with HAdV were found 16 and 12.85% before MLS administration and were found 14.6 and 10.4% respectively after MLS administration. Necrosis ratio of HEp-2 and Vero cells infected with HAdV were found 34 and 28.2% before MLS administration and were found 30 and 26.4% respectively after MLS administration (Figure 2).

The tissue culture infectious doses ($TCID_{50}$) of HSV-1 and HAdV for HEp-2 cells and Vero cells were higher in the cells treated with diluted montelukast. The $TCID_{50}$ values of HSV-1 for Hep-2 cells and Vero cells with no MLS were $10^{-5,25}/0.1$ and $10^{-4,95}/0.1$ ml, respectively. The $TCID_{50}$ values of HSV-1 for Hep-2 cells and Vero cells diluted with 9.76 µg/ml MLS were $10^{-4,15}/0.1$ and $10^{-3,75}/0.1$ ml, respectively (Figure 3). The $TCID_{50}$ values of HAdV for Hep-2 cells and Vero cells with no MLS were $10^{-7,0}/0.1$ and $10^{-6,75}/0.1$ ml, respectively. The $TCID_{50}$ values of HAdV for Hep-2 cells and Vero cells diluted with 9.76 µg/ml MLS were $10^{-6,25}/0.1$ and $10^{-5,75}/0.1$ ml, respectively (Figure 4).

DISCUSSION

In the present study, we investigated the changes in $TCID_{50}$ values and cellular death responses of herpes simplex virus type-1 (HSV-1) and HAdV-infected HEp-2 and Vero cells to non-toxic doses of montelukast.

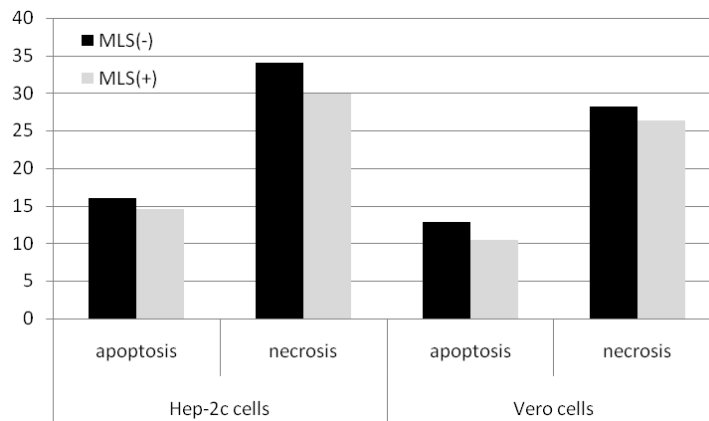Physiological cell death kills unwanted host cells and

Figure 2. Apoptotic/necrotic behaviors of cells infected with HAdV with/without montelukast.
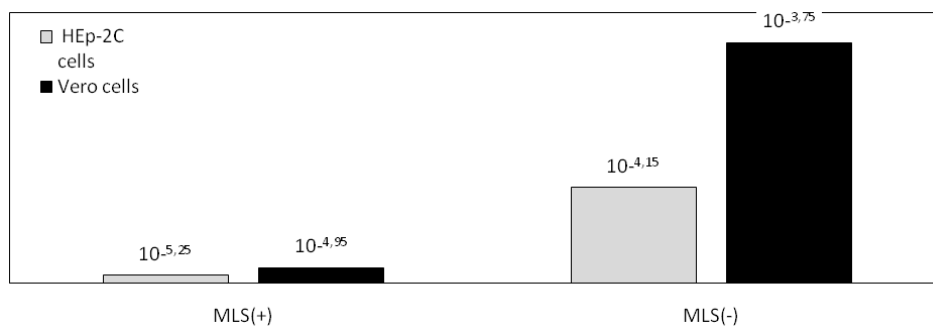

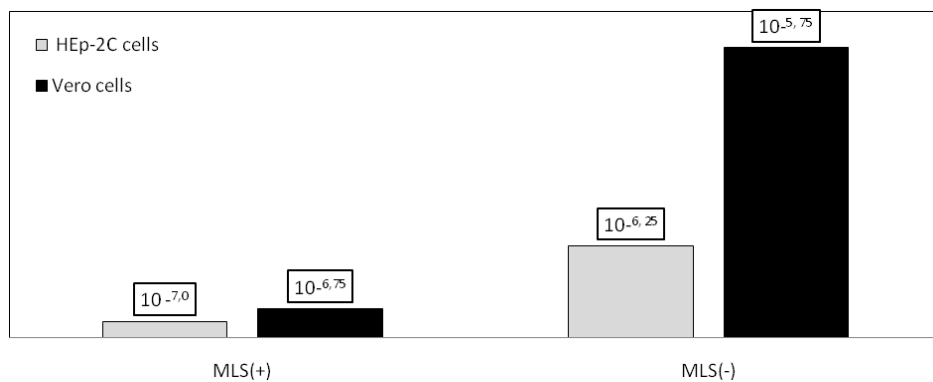**Figure 3.** $TCID_{50}$ values of HSV1 infected cells with/without montelukast.

Figure 4. $TCID_{50}$ values of HadV infected Hep2-C and Vero cells with/without montelukast

also occurs in aging. This process of cell suicide is called apoptosis (Johnston et al., 2005), and healthy cells have a basal apoptosis/necrotic turnover rate. At a high concentration, montelukast itself has apoptotic/necrotic effects. In our study, the maximum non-toxic dose of montelukast for healthy cells was first determined in order to prevent such a potential bias; the non-toxic dose was 9.76 µg/ml.

A variety of stimuli can also provoke apoptotic cell death, among them metabolic disturbances due to drugs, chemical insults or viral infections. Several viral gene products affect apoptosis by directly interacting with the components of highly conserved biochemical pathways that regulate cell death. It appears that a growing number

of viruses may lead to the culmination of a lytic infection and serve to spread viral progeny to neighbouring cells whilst evading inflammatory responses of the host.

In such a case, apoptosis could contribute to the cytotoxicity associated with viral infections. The aim of the present study was to observe apoptotic and necrotic changes in cells before and after the administration of montelukast to determine its antiviral activity.

We found that there were fewer apoptotic and necrotic HEp-2 and Vero cells infected with HSV-1 after the administration of diluted montelukast ($p<0.05$) (Figure 1). Similarly, there were fewer apoptotic and necrotic HEp-2 and Vero cells infected with HAdV after the administration of diluted montelukast ($p<0.05$) (Figure 2).

Although these results suggest the existence of a possible relationship between the apoptotic/necrotic response and montelukast, they cannot be taken to mean that a decrease in apoptotic/necrotic activity implies protection against viral infection. The role of viruses in apoptosis is like a game in which different teams play at the same time; thus, whether the virus actively induces apoptosis and, subsequently, cell death, or whether the infected cell falls becomes apoptotic to limit viral replication is unknown.

In an attempt to elucidate this relationship, in our study we also evaluated the effect of montelukast on the $TCID_{50}$ values of the viruses for HEp-2 and Vero cells. We found that the tissue culture infectious dose ($TCID_{50}$) values of HSV-1 and HAdV for HEp-2 cells and Vero cells were higher in cells treated with diluted montelukast, and this result clearly showed that the viral infectivity dramatically decreased after montelukast administration, similar to the changes in the apoptotic/necrotic response (Figures 3 and 4).

When we take all of our results into consideration, our data suggest a possible antiviral effect of montelukast. Our study is the first one to evaluate the antiviral activity of montelukast at a cellular level *in vitro*.

The inflammatory response to allergic diseases such as asthma, atopic dermatitis and allergic rhinoconjunctivitis induces the production of leukotrienes. Cohort studies have shown that viruses are probably the most common cause of asthma exacerbations (Johnston et al., 2005; Nıcholson et al., 1993). Adenovirus has the potential to cause acute asthma attacks in children at any age, and persistent adenovirus has been found in the lower respiratory track of children with a history of wheezing following acute adenovirus bronchiolitis (Macek et al., 1994; Tan et al., 2003). Adenovirus is capable of lysing infected cells during the viral replicative cycle, and it can directly initiate an inflammatory response by damaging host tissues. As our data suggested that montelukast might decrease the lytic capability of this virus, the hypothesis that montelukast has an antiviral effect, or at least an anti-adenoviral effect, and that this capability might be important in the control of asthmatic symptoms, is not wrong.

The epithelium lining the airways is a physical barrier, and a protective barrier, that separates the airways from the external environment. The epithelium produces many pro- and anti-inflammatory substances (Woolcock and Barnes, 1992; Thompson, 1998; Holgate et al., 1999). Bronchial epithelial cells are a target for viral pathogens and a site of viral infection and replication. Cellular responses and damage to epithelial cells upon viral infection are known to be involved in, and possibly trigger, the acute exacerbation of bronchial asthma (Wark and Gobson, 2006). Virus-induced damage to the epithelium most likely involves apoptosis and necrosis of host cells; apoptosis can be seen as an acute anti-viral inflammatory response. Damage to the airway epithelium by viruses not only compromises its protective barrier function but also results in the decreased production of smooth muscle relaxant factors and the increased release of pro-inflammatory cytokines such as IL-6 and TNF-alpha. Because of this repeated damage and repair, the epithelium becomes altered and over-produces mucus, growth factors and pro-inflammatory cytokines such as TNF-alpha, which act to further aggravate inflammation and increase airway hyperresponsiveness (AHR) (Woolcock and Barnes, 1992; Holgate et al., 1999). While apoptosis of damaged airway epithelium cells is likely to be beneficial in the normal respiratory tract, the excessive and inappropriate apoptosis of absolute eosinophil count (AEC) may contribute to the pathogenesis of chronic inflammatory respiratory disease. Therefore, factors or drugs, possibly including MLS, which increase the resilience of the airway epithelium to apoptosis, are likely to decrease the severity of this disease. Unfortunately, there have been no clinical trials on this; a general literature search about the relationship between anti-leukotrienes, asthma and viruses only found a few articles and almost all of them were about respiratory synctial virus (RSV) and montelukast.

There are a few studies about the relationship between LTRA and viral infections in asthmatic patients. It was suggested that MLS decreased the rate of asthma exacerbation (Bisgaard et al., 2005). In our previous study, we also found that MLS decreased the rate of asthma attacks (Igde and Anlar, 2009). When we take into consideration the fact that viral infections account for 85% of childhood asthma exacerbations, daily symptoms and exacerbations of asthma in children (Johnston et al., 1995) and adults (Nicholson et al., 1993), the possible protective effect of MLS against viral infections may be the explanation for such a decrease in the rate of asthmatic attacks in children. Studies have been performed on the anti-RSV virus effect of MLS, but unfortunately their results are inconsistent. Some reported a protective effect of MLS against RSV (Bisgaard 2003; Szefler et al., 2003; Proesmans et al., 2009), while others suggested the opposite situation (Han et al., 2010; Kim et al., 2010).

Different explanations are possible for such conflicting results. First, for our hypothesis to be effective, MLS should be given before airway epithelium desquamation; none of the studies considered this fact and, because the effectiveness of MLS against viral infections could be influenced by the stage of the disease, this might lead to different results and it might cause biases. Secondly, the results of recent studies support the hypothesis that host factors could determine the pattern of RSV disease (De Vincenzo, 2005). This situation may be generalized as different viruses with different mechanisms, even for same viruses with possible different host factors, may lead to different host responses to MLS treatment in asthmatic patients against viral infections. But none of these works studied different viruses or different host subgroups.

Kaposi varicelliform (KV) eruption is the name given to a distinct cutaneous eruption. It is most commonly caused by a disseminated HSV infection in patients with another important allergic disease/atopic dermatitis and for this reason it is often referred to as eczema herpeticum (Schroeder et al., 2009). Although to date the pathophysiology of Kaposi varicelliform eruption remains unclear, we know that herpes is an important leading viral agent of KV, and our data support a possible anti-HSV effect of MLS, thus MLS may be protective against KV in children with atopic dermatitis. Unfortunately, no study was found that researched the protective effect of MLS against KV in patients with atopic dermatitis.

Although the methods applied in this study mostly provide opportunities for a morphological diagnosis, we believe that our results are interesting because there have not been any similar studies that directly evaluated the relationship between MLS and viral pathogens *in vitro*. Furthermore, these results are also important for explaining how the possible antiviral effect of MLS, which is used widely, especially in allergic diseases, may happen. In order to be sure about this, new studies that include molecular methods to explain the pathogenic mechanisms at the cellular level are needed. Also, because viral pathogens have different pathogenic mechanisms, studies related to other viral agents, like RSV and Rhino viruses, which are important in allergic diseases, are needed both *in vitro* and *in vivo*.

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