

## In Vitro Caco-2 Cell Permeability Studies of Ziprasidone Hydrochloride Monohydrate Nanocrystals

### Ziprasidon Hidroklorür Monohidrat Nanokristallerinin İn Vitro Caco-2 Hücre Permeabilite Çalışmaları

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#### ÖZET

**Amaç:** Bu çalışma, ziprasidone hidroklorür monohidrat (ZHM) nanokristallerinin Caco-2 hücreleri üzerindeki sitotoksik etkisini ve geçirgenliğini değerlendirmeye odaklanmıştır.

**Gereç ve Yöntemler:** ZHM nanokristalleri, stabilizan olan PVP varlığında mikrofluidizasyon yöntemi ile hazırlanmıştır. Partikül büyüklüğü (PB), partikül büyüklüğü dağılımı (PBD) ve zeta potansiyel (ZP) değerleri karakterizasyon çalışmaları kapsamında ölçülmüştür. ZHM nanokristallerinin in vitro sitotoksik etkisi MTT testi ile araştırılmıştır. Caco-2 hücrelerinden geçiş çalışmaları, ZHM kaba tozu ve nanokristal formülasyonları ile gerçekleştirilmiştir.

**Bulgular:** ZHM nanokristalleri 400-600 nm PB, 0.1-0.4 PBD ve >20 mV ZP değerleri ile elde edilmiştir. Tüm çalışma gruplarında hücre canlılığı %100 kalmıştır. Caco-2 hücrelerinden geçiş çalışmalarında, ZHM nanokristalleri, permeabilite değerini ZHM kaba tozuna kıyasla 2.3 kat artırmıştır. Aynı zamanda kümülatif geçen ilaç miktarı örnek alma süresinin sonunda yükselmiştir.

**Sonuç:** Nanokristal teknolojisinin doymun çözünürlüğü artırmasına bağlı olarak permeabiliteyi artırmada yarar sağlayabileceği sonucuna varılmıştır.

**Anahtar Kelimeler:** Caco-2 hücreleri, permeabilite, ziprasidone, nanokristal

#### ABSTRACT

**Objectives:** The current study focused on the evaluation of the cytotoxic effect and permeability of ziprasidone hydrochloride monohydrate (ZHM) nanocrystals on Caco-2 cells.

**Materials and Methods:** ZHM nanocrystals were prepared by microfluidization method in existence of PVP as stabilizer. Particle size (PS), particle size distribution (PDI), and zeta potential (ZP) values were measured regarding the characterization studies. In vitro cytotoxic effect of ZHM nanocrystals was investigated using MTT test. Caco-2 transport studies were conducted with ZHM coarse powder and nanocrystal formulation.

**Results:** The nanocrystals were obtained with 400-600 nm PS, 0.1-0.4 PDI, and >20 mV ZP values. The cell viability remained 100% for all sample groups. The permeability value of ZHM nanocrystal through Caco-2 cells increased 2.3-fold in comparison with ZHM coarse powder. Cumulative transported drug also increased at the end of the sampling time.

**Conclusion:** It has been concluded that nanocrystal technology helps to increase permeability of drug particles in terms of increasing the saturation solubility.

**Key words:** Caco-2 cells, permeability, ziprasidone, nanocrystal

## INTRODUCTION

The new drug candidates are coming with poorly water soluble properties, which causes insufficient bioavailability by limiting absorption (Merisko-Liversidge and Liversidge, 2008). Saturation solubility depends on the radius of the particles according to Ostwald-Freundlich equation (Eq. 1) (Scholz et al., 2014; Wu and Nancollas, 1998).

$$\ln\left(\frac{S}{S_0}\right) = 2 M \gamma / \rho r R T \quad \text{Eq.1}$$

Where, S is the solubility, r is the radius of the particles, S<sub>0</sub> is the normal solubility value (plane surface), M is the molecular weight,  $\gamma$  is the interfacial tension,  $\rho$  is the density, R is the gas constant, and T is the temperature (Kelvin).

Reducing particle size to nanometer range can increase the surface area which leads to an increase in kinetic saturation solubility and dissolution velocity according to Kelvin and Noyes-Whitney equations. Membrane penetration and finally enhanced bioavailability can be achieved by particle size reduction (Karakucuk and Celebi, 2020; Zhai et al., 2014).

Nanocrystals are 100% drug molecules with <1000 nm particle size (typically 200-600 nm) (Keck and Müller, 2006). They consist of a minimum amount of stabilizer such as polymer and/or surfactants (Gao et al., 2012). Because of the advantage with regard to particle size, they increase saturation solubility and hence the permeability and dissolution rate of the drug component which cause increased bioavailability.

Ziprasidone Hydrochloride Monohydrate (ZHM), an antipsychotic drug, is a Biopharmaceutical Classification System (BCS) Class II drug which has low water solubility and high permeability (Zakowiecki et al., 2015). The absorption of ZHM is affected by the existence of food (Thombre et al., 2012). The dissolution-rate limited performance causes highly variable bioavailability and absorption profile which is affected by fed/ fasted state of the patient (Patravale et al., 2004). Preparing the nanocrystals of ZHM can increase saturation solubility and hence dissolution rate, which result in eliminating the food effect due to drug absorption, and enhancing permeability and bioavailability of ZHM (Ghosh et al., 2011; Karakucuk et al., 2019; Kesisoglou et al., 2007).

Intestinal drug absorption is affected by the permeability of drugs, and there are several methods for investigating permeability during drug development process (Lennernas, 1998). One of the methods is monolayers of cultured suitable cells, which is recommended by Food and Drug Administration for determining drug substance permeability. Human colon epithelial cancer cell line (Caco-2 cell line) is used as an in vitro model to predict drug permeability. Caco-2 cells are differentiated to mimic small intestinal epithelium when cultured in conventional culture conditions as monolayers (Artursson et al., 2012). It has been shown that there is an excellent correlation between in vivo absorption and in vitro apparent

permeability which is obtained from Caco-2 cell model (Artursson et al., 2012; Yee, 1997). Besides compound screening in high throughput format during discovery phase, Caco-2 model can be used to investigate the formulation effect on permeability. Some excipients can compromise tight junction integrity or cause changes in efflux system and increase permeability (Rege et al., 2001); therefore, it is important to investigate formulation permeability during drug development.

Previous studies showed that saturation solubility was dramatically enhanced with nanocrystal formulation of ZHM (Tashan et al., 2019), and orally disintegrating tablet form was developed successfully (Tashan et al., 2020). This study focused on the in vitro cytotoxicity as well as Caco-2 cell permeability of ZHM nanocrystals and the effect of particle size on permeability was investigated.

## **MATERIALS AND METHODS**

### *Materials*

Ziprasidone hydrochloride monohydrate (ZHM) was kindly gifted by Abdi Ibrahim Pharmaceuticals (Istanbul, Turkey). Polyvinylpyrrolidone K30 (PVP K30), was purchased from Sigma Aldrich (USA). Human epithelial colorectal adenocarcinoma cell (Caco-2, ATCC HTB 37) was obtained from ATCC. Hank's Balanced Salt Solution (HBSS), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), Dulbecco's Modified Eagle Medium (DMEM) were purchased from Sigma Aldrich (USA). Other chemicals for HPLC were analytical grade.

### *HPLC analysis of ZHM*

The ZHM concentration was determined using HPLC method. Agilent 1200 HPLC (Agilent Technologies, California, USA) system equipped with an autosampler and a UV-Vis detector was used for this purpose. The concentration range was 12- 20 µg/mL. The analytical column was ODS C18 (RP) Column (150 mm x4.6 mm, 5 µm) (TSKgel) for sample separation. The mobile phase consisted of potassium dihydrogen phosphate (6.8 g/L and with pH 3.0 o-phosphoric acid) 10:90 (v/v). Flow rate was 1 mL/min, injection volume was 20 µL, and column temperature was 25 °C. The detection wavelength was 229 nm and retention time was 2.4 minute.

### *Preparation of ZHM nanocrystals*

The ZHM nanocrystals were prepared according to a previous study (Tashan et al., 2019). Microfluidization technique (Microfluidics LV1, Microfluidizer® Processors, USA) was used to obtain nanocrystals. For this purpose, 0.5% (w/w) ZHM was dispersed into the 0.5% (w/w) PVP K30 solution. Macro suspensions were stirred under the homogenizer (Ultraturrax, Heidolph, Germany) at 15 000 rpm for 10 minutes. Microfluidization method was performed at 30 000 psi pressure for 20 cycles. Particle size, particle size distribution, and zeta potential values were measured using Malvern ZetaSizer (Malvern Instruments, UK).

ZHM amount in ZHM nanocrystals was analyzed with validated UV spectrophotometric method. Lyophilized nanocrystals were dissolved completely in methanol, filtered through 0.45 µm membrane filter, and then measured at 314 nm wavelength.

### *Cell Culture Studies*

The cell culture studies were conducted on Human epithelial colorectal adenocarcinoma cells (Caco-2) to determine the permeability values of the ZHM coarse powder and ZHM nanocrystals. The effect of the sample groups on Caco-2 cell viability was investigated with MTT test.

### *Preparation of the Caco-2 cells*

Firstly, the Caco-2 cells were removed from -180 °C nitrogen tank and thawed in water-bath at 37 °C. The Caco-2 cells in vial were transferred into the culture media of 15 mL under laminar flow. The media was centrifuged for 3 minutes at 2000 rpm and supernatant was removed. The precipitated cells were re-suspended and were transferred into 25 cm<sup>2</sup> flasks. The cells were incubated at 37 °C, in air containing 5% CO<sub>2</sub>, in the Dulbecco's Modified Eagle's Medium (DMEM) with 10% (v/v) fetal bovine serum (FBS), 2mM L-glutamine, 50 IU/mL penicillin and 50 µg/mL streptomycin. The media was changed every other day. After 6-7 days, when the cell frequency reached 80-90%, the cells were treated with trypsin-EDTA (0.25%) to remove cells from flask surface, then cells were transferred to other flasks. Cells with passage 24 were used for permeability studies.

### *Transferring Caco-2 cells into inserts*

After 24 passage cycle, the cells were counted using microscopy with hemocytometer using trypan blue. The suspended cells were seeded into 6-well plate inserts (Snapswell™, 6 well, 0.4 µm pore diameter) at a density of 70.000 cells per well. 1.5 mL and 0.5 mL media were put into the basolateral and apical sides, respectively. The culture medium was changed every other day for 21 days. The photo of the cells in the culture media was taken with inverted microscope (Pic 1).



(a) (b)  
Pic 1. Photo of the inserts in the inverted microscope. (a) Empty inserts; (b) Caco-2 cells

### *In vitro cytotoxicity*

MTT cell viability test was executed to investigate the effect of the coarse powder of ZHM and nanocrystals on Caco-2 cells and to determine the concentrations to be applied in permeability study (Wang et al., 2019). Caco-2 cells were seeded into the 96-well plates at a density 5000 cell/well. The ZHM coarse powder and ZHM nanocrystals were dissolved in DMSO or dispersed in culture media (DMEM) and added into the 96-well plates. 0.4% DMSO was added as control group. The plates were incubated at 37 °C, in air containing 5% CO<sub>2</sub> for four hours, which was the duration for permeability studies. After incubation period, 25 µL of MTT (5 mg/mL) solution was added to the wells and plates were further incubated for four hours. Then, the media in the wells were removed, 200 µL of DMSO was added to each well, and absorbance was read using microplate reader at 570 nm to measure optical density. Cell viability was calculated according to Eq. 2.

$$\text{Cell viability (\%)} = (\text{Ab}_{\text{test}}/\text{Ab}_{\text{control}}) \times 100 \quad \text{Eq.2.}$$

### *Evaluation of monolayer integrity of the cells*

The transepithelial electric resistance (TEER) of the monolayer Caco-2 cells was measured with Millicell-ERS voltohmmeter. Monolayers with TEER > 300  $\Omega$ .cm<sup>2</sup> were used for permeability studies (Shekhawat et al., 2019).

TEER values were calculated according to Eq. 3, where R<sub>sample</sub> is the resistance of inserts which contains cells; R<sub>empty</sub> is the resistance of inserts which are empty; A is the surface area of the cell culture inserts (cm<sup>2</sup>).

$$\text{TEER}_{\text{cell layer}} = (R_{\text{sample}} - R_{\text{empty}}) \times A \quad \text{Eq. 3.}$$

#### *In vitro permeability*

The HBSS buffer with 10 mM Hepes (pH 7.4) was used for the in vitro permeability studies. The samples were prepared in 0.4% DMSO containing HBSS. Stock ZHM concentration of the formulations was 100  $\mu$ g/mL. The other concentrations were diluted from stock solution. The stock concentration was selected according to the oral ZHM dose.

Firstly, the culture media in the basolateral and apical sides were removed and these compartments were treated with HBSS. After 30 minutes of incubation, whole medium was removed. 0.5 mL of the sample was put into the apical compartment and 1.5 mL of the HBSS buffer was put into the basolateral compartment (n=6). The plates were incubated at 37 °C, at 60 rpm for 4 hours. The sampling intervals were 30, 60, 90, 120, 240 minutes. At the sampling time points, 0.5 mL sample was withdrawn and fresh HBSS buffer was added to the basolateral side. The samples were analyzed using validated HPLC method. The apparent permeability coefficient (P<sub>app</sub>, cm/s) was calculated according to Eq. 4.

$$P_{\text{app}} = dC/dt \times 1/(A \times C_0) \quad \text{Eq. 4.}$$

Where, dC/dt is the drug permeation rate ( $\mu$ g/s); A is the surface area of the inserts (cell monolayer) (cm<sup>2</sup>); C<sub>0</sub> is the initial concentration at the apical side ( $\mu$ g/mL).

## **RESULTS AND DISCUSSION**

### *Preparation of Nanosuspensions*

Microfluidization is one of the top-down methods to produce nanocrystals. The main advantages of this method are the repeatability of experiments, easy to scale up and relatively fewer process validation parameters (Karakucuk et al., 2016).

In this study, ZHM nanocrystals characterized with 532.4  $\pm$  13.7 nm particle size, 0.304  $\pm$  0.01 particle size distribution, and 20.5  $\pm$  0.3 mV zeta potential values were used for cell culture studies. The ZHM amount was found 95%  $\pm$  5% for the nanocrystals according to validated UV spectrophotometric method (Tashan et al., 2019).

### *Cell Culture Studies*

#### *Caco-2 Cell Viability*

MTT assay was conducted to understand the in vitro toxicity of the coarse ZHM and ZHM nanocrystals. Cell viability (%) was determined after the interaction between Caco-2 cells and ZHM formulations for four hours. ZHM coarse powder or ZHM nanocrystals were not cytotoxic even at the highest dose, which was 200  $\mu$ g/mL. 0.4% DMSO was used to dissolve ZHM particles and applied to the cells as control group. No cytotoxic effect was observed for the DMSO at this concentration (Fig. 1). As ZHM dose for the treatment is 20 mg and considering the drug intake with one glass of water, which is a volume of 200 mL, the permeability studies continued with 100  $\mu$ g/mL concentration. In literature, it was reported previously that PVP K30 polymer was not cytotoxic at the concentration we used (He et al., 2014).

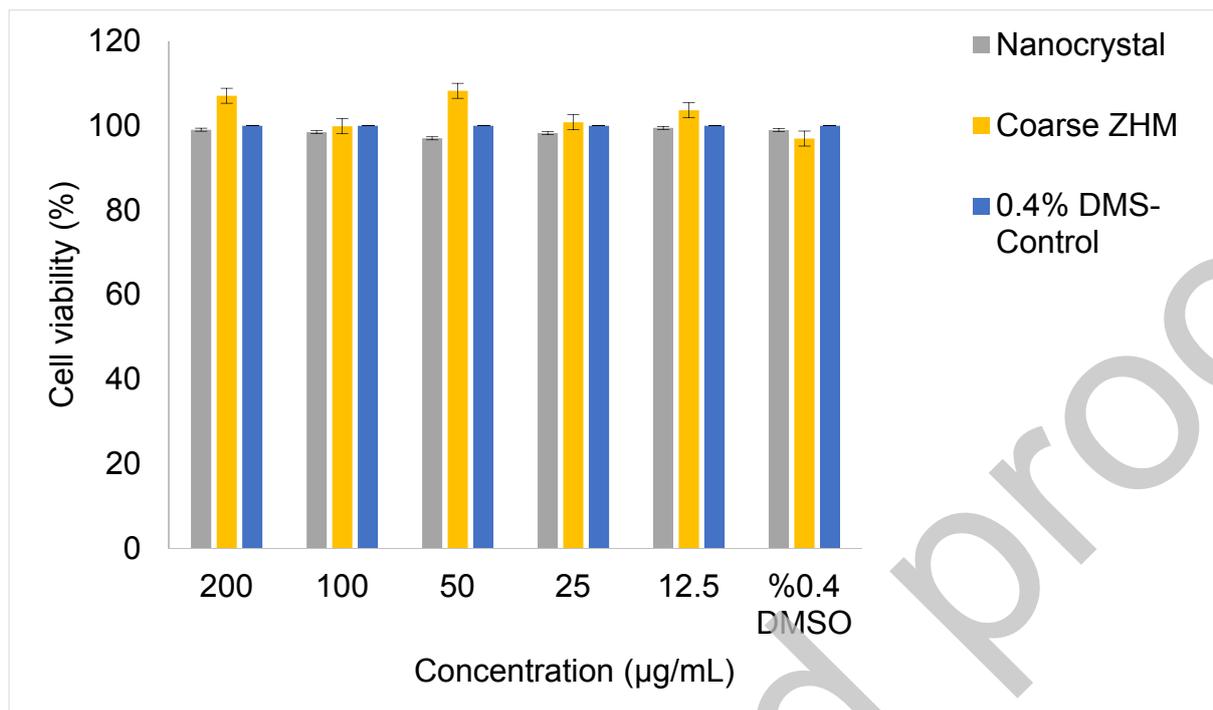


Fig. 1. Cell viabilities (%) of the sample groups (n=6)

#### *Caco-2 Cell Permeability*

Saturation solubility, dissolution rate and drug permeability are crucial factors for improving oral bioavailability. It is well known that dissolution rate can be increased by reducing particle size of drug substance to sub-micron region, due to increased surface area. Nanocrystal technology taking advantage of this phenomenon is a useful method for improving bioavailability of poorly soluble compounds (Jinno et al., 2006). Besides in vitro solubility and dissolution studies, nanocrystal formulations should be tested regarding their permeability to investigate the influence of particle size on drug permeability and to complement solubility and dissolution studies (Hecq et al., 2006). In this regard, the permeability of nanocrystal formulation was investigated across Caco-2 cell monolayer. Monolayer integrity of Caco-2 cells was showed with TEER values of 300-1000 ohm.cm<sup>2</sup> which is in accordance with literature (Wahlang et al., 2011). The permeability value of coarse ZHM was found  $8.887 \times 10^{-6}$  cm/s at the end of four hours. The permeability value increased 1.34-fold with nanocrystal formulation with the value of  $11.931 \times 10^{-6}$  cm/s (Fig. 2). In the previous study, it was shown that ZHM nanocrystals increased saturation solubility for 2.3-fold in comparison with the coarse powder of ZHM (Tashan et al., 2019). The increase of the permeability value can be explained by the increase in saturation solubility by nanocrystal formulation (Gulsun et al., 2018). Reduced particle size of nanocrystal formulation increases saturation solubility of ZHM; therefore, the amount of dissolved ZHM in apical side is greater for nanocrystal formulation compared to coarse powder (Hecq et al., 2006). Hence, this results in an increased permeation rate for ZHM nanocrystals.

ZHM is known as a BCS Class II drug which has low water solubility as well as high permeability (Patil et al., 2012; Zakowiecki et al., 2015). Log P value of ZHM is 3.6, and the drugs which have Log P value between 2.9- 5.2 have expected permeability value of  $10^{-5}$ -  $10^{-4}$  (Paixão et al., 2010). The drugs, which are completely absorbed at Caco-2 monolayers, have high permeation coefficient. Permeability values can be classified as  $P_{app} < 1 \times 10^{-6}$  cm/s,  $> 10 \times 10^{-6}$  cm/s and between  $1-10 \times 10^{-6}$  cm/s for the drugs which have low, high, and moderate

permeability properties, respectively (Yee, 1997). Considering this information, nanocrystal formulation increased the permeability value of ZHM from moderate to high.

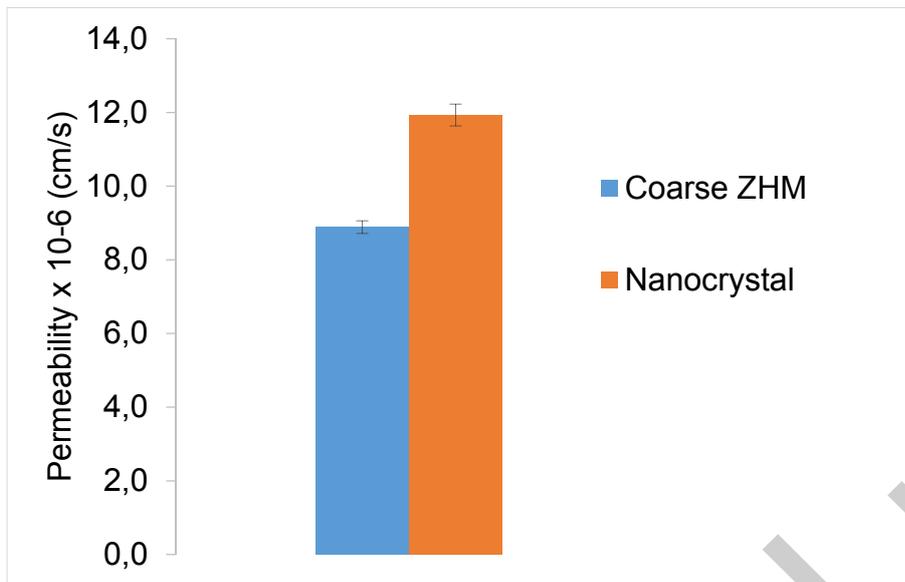


Fig. 2. The permeability values of coarse ZHM and nanocrystal

The cumulative transported drug was also found higher with nanocrystal formulation in comparison with coarse powder which is considered to be related to improved solubility of ZHM in nanocrystal form (Fig. 3).

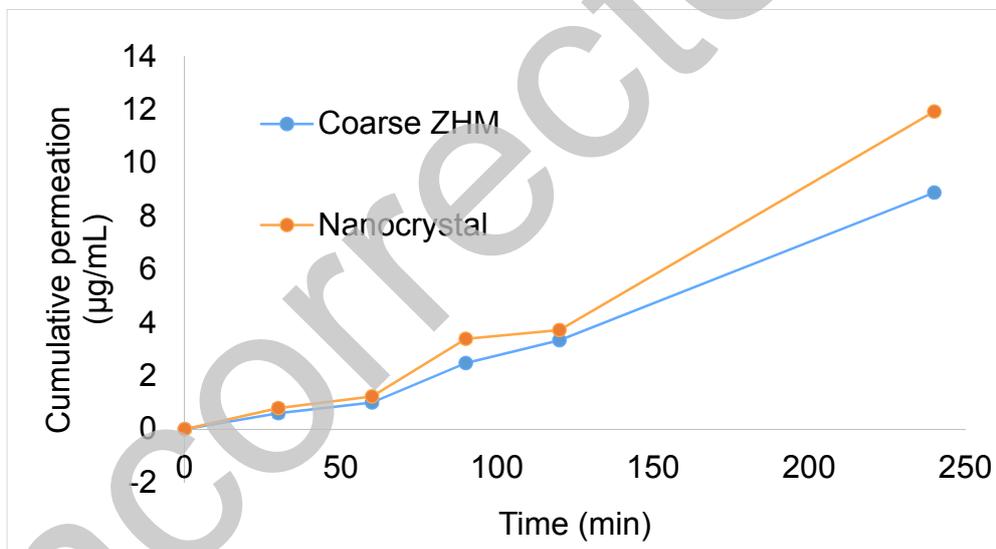


Fig. 3. Cumulative penetrated ZHM amount in basolateral membrane

## CONCLUSION

The microfluidization method was found to be an effective and easy technique to prepare nanocrystal formulations. ZHM nanocrystals were successfully obtained, lyophilized, and applied to Caco-2 cells. Coarse powder of ZHM or nanocrystals had no toxic effects on the Caco-2 cells regarding the applied dose. ZHM nanocrystals showed enhanced permeability value in comparison with the coarse powder. In addition, cumulative penetrated drug amount reached a higher concentration by nanocrystals. In light of these results, it can be concluded that nanocrystal formulations can enhance permeability of the drug substance.

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