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RESEARCH ARTICLE

Antiviral activity of Basidiomycete mycelia against influenza type A (serotype H1N1) and herpes simplex virus type 2 in cell culture

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In this study, we investigated the *in vitro* antiviral activity of the mycelia of higher mushrooms against influenza virus type A (serotype H1N1) and herpes simplex virus type 2 (HSV-2), strain BH. All 10 investigated mushroom species inhibited the reproduction of influenza virus strain A/FM/1/47 (H1N1) in MDCK cells reducing the infectious titer by 2.0–6.0 lg ID_{50}. Four species, *Pleurotus ostreatus*, *Fomes fomentarius*, *Auriporia aurea*, and *Trametes versicolor*, were also determined to be effective against HSV-2 strain BH in RK-13 cells, with similar levels of inhibition as for influenza. For some of the investigated mushroom species—*Pleurotus eryngii*, *Lyophyllum shimeji*, and *Flammulina velutipes*—this is the first report of an anti-influenza effect. This study also reports the first data on the medicinal properties of *A. aurea*, including anti-influenza and antiherpetic activities. *T. versicolor* 353 mycelium was found to have a high therapeutic index (324.67), and may be a promising material for the pharmaceutical industry as an anti-influenza and antiherpetic agent with low toxicity. Mycelia with antiviral activity were obtained in our investigation by bioconversion of agricultural wastes (amaranth flour after CO_2 extraction), which would reduce the cost of the final product and solve some ecological problems.

KEYWORDS antiviral activity; Basidiomycetes; mycelium; influenza A virus (H1N1); herpes simplex virus type 2 (HSV2)

INTRODUCTION

The prevention and treatment of viral diseases remains an urgent problem in modern medicine. Influenza virus infection is one of a challenging global issues with regular widespread epidemics or pandemics resulting in high mortality worldwide. The type A viruses are the most virulent human pathogens among the three influenza types and cause the most severe disease. A total of 18,500 laboratory-confirmed deaths were caused by the 2009 influenza A (H1N1) pandemic in the period April 2009–August 2010, but estimates of respiratory and cardiovascular mortality associated with this pandemic were 15 times higher than reported laboratory-confirmed deaths (Dawood F S, et al., 2012). Sexually transmitted diseases represent another global healthcare problem. Worldwide, many people are infected with herpes si-mplex virus type 2 (HSV-2; a sexually transmitted disease that can cause recurrent, painful genital ulcers). Furthermore, HSV-2 is potentially lethal for neonates (Corey L, et al., 1988) and may facilitate the transmission of the human immunodeficiency virus (Holmberg S D, et al., 1988). The importance of the search for effective antiviral medications, including those of natural origin, is obvious.

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The biologically active substances of Basidiomycetes—their fruit bodies, vegetative mycelia (biomass), spores, or cultural liquids, deprived of side effects—may form the basis of such drugs. A number of mushroom species have been identified as potential sources of drugs, with antiviral action against the influenza virus (Mothana R A A, et al., 2003; Ohta Y, et al., 2007; Ibragimova Zh B, et al., 2012; Vlasenko V A, et al., 2012; Fillipova E I, et al., 2012, 2013) and HSV-2 (Amoros M, et al., 1997; Oh K.-W, et al., 2000; Kostina N E, et al., 2013). Fruiting bodies have been the object of most studies of the antiviral activity of Basidiomycetes (Takehara M, et al., 1979; Amoros M, et al., 1997; El-Mekkawy S., et al., 1998; Eo S-K, et al., 1999; Pirano F, et al., 1999; Wang H X, et al., 2000; Oh K.-W, et al., 2000; Awardh A N A, et al., 2003; Mothana R A A, et al., 2003; Ngai P H K, et al., 2003; Stamets P, 2005; Bruggemann R, et al., 2006; Ohta Y, et al., 2007; Faccin L C, et al., 2007; Gu C Q, et al., 2007; Lv H, et al., 2009; Kabanov A S, et al., 2011; Ibragimova Zh B, et al., 2012; Vlasenko V A, et al., 2012; Fillipova E I, et al., 2012, 2013; Kostina N E, et al., 2013). However, the vegetative mycelium of these mushrooms is not inferior to fruit bodies in terms of the content of antiviral active substances (Hirose K, et al., 1987; Eo S-K, et al., 2000; Liu J, et al., 2004; Ng T B, et al., 2006; Cardozo F T G S, et al., 2011; Prozenko M A, et al., 2012; Teplyakova T V, et al., 2012) and has a number of advantages in the cultivation process. Vegetative mycelium has a constant qualitative and quantitative composition, and its cultivation requires much less time and significantly less energy. Another little-studied aspect is the influence of the substrate on the antiviral activity of mycelium. Only Teplyakova T V, et al. (2012) have used a non-standard substrate (oat-corn water) in investigating the antiviral activity of polyporoid mushroom mycelium.

The aim of the current work was to assess the antiviral activity of extracts from Basidiomycetes mycelium, cultivated on a natural substrate (amaranth flour after CO_2 extraction) in *in vitro* assays against influenza virus type A and HSV-2.

MATERIALS AND METHODS

Mushrooms

The mushroom species *Auriporia aurea* 5048 (Peck) Ryvarden, *Flammulina velutipes* 1878 (Curtis) Singer, *Fomes fomentarius* 355 (L.) Fr., *Ganoderma lucidum* 1900 (Curtis) P. Karst., *Lentinus edodes* 502 (Berk.) Singer, *Lyophyllum shimeji* 1662 (Kawam.) Hongo, *Pleurotus eryngii* 2015 (DC.) Quel., *Pleurotus ostreatus* 551 (Jacq.) P. Kumm., *Schizophyllum commune* 1768 Fr., and *Trametes versicolor* 353 (L.) Lloyd. were kindly supplied by the Culture Collection of Mushrooms (IBK) of the M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine (Buchalo A S, et al., 2011).

The waste of flour from *Amaranthus hybridus* L. (variety "Ultra"; Mykolaiv Region, Ukraine, 2011) grains after CO_2 extraction was used as the base of the culture medium. CO_2 extraction conditions were as follows: pressure 7.2 MPa; temperature 24 ºC; time of extraction 2 h. Mycelial cultures were initially grown in Petri dishes (90 mm diameter) on culture medium with pH 6.0, composed of (g/L): 20.0 glucose, 3.0 yeast extract, 2.0 peptone, 1.0 K_2HPO_4, 1.0 KH_2PO_4, and 0.25 $MgSO_4{\bullet}7H_2O$. The liquid culture medium (substrate-60 g amaranth flour in 1 L distilled water) was sterilized by autoclaving for 20 min at 121 ºC. Flasks (250 mL) with 50 mL liquid medium were inoculated with three mycelial plugs of 8 mm diameter cut from the Petri dishes using a sterile borer at the stage of actively growing mycelia. Mycelia were grown as static cultures in flasks for 14 days at 26 ± 2 ºC.

Extracts from mushroom mycelia (biomass)

The samples used in experiments were of mycelial extracts. Biomass was separated from the culture liquid, thoroughly washed with distilled water, dried with high-vacuum freeze drying with a Cryodos-50 freeze dryer (Terrasa, Spain), and pounded in a porcelain mortar. A total of 300 mg biomass was suspended in 3 mL sterile 0.9 % NaCl solution and sonicated using an MSE-100 W sonifier (MSE, London, UK) for 20 min at amplitude 24 µm. The precipitate was separated using a Beckman Coulter J2-21 centrifuge (Beckman Coulter, Inc, Brea, CA, USA) for 20 min at 10,000 rpm, and the supernatant was used for studies. The extracts (samples) were refrigerated at -20 ºC.

Cell lines and viruses

MDCK (Madin–Darby canine kidney) and RK-13 (rabbit kidney) cells were grown in medium containing 90% RPMI 1640 medium (R8758, Sigma-Aldrich, Schnelldorf, Germany) supplemented with 10% fetal bovine serum (F7524, Sigma-Aldrich), penicillin (100 U/mL), streptomycin (100 U/mL), and kanamycin (50 U/mL). Cell cultures were maintained at 37 ºC in a humidified atmosphere with 5% CO_2. The test viruses included influenza virus strain A/FM/1/47 (H1N1) (the infectious titer in MDCK cells was 10^6 median tissue culture infective dose [$TCID_{50}$]/mL, hemagglutination titer 1:256) and HSV-2 strain BH (infectious titer 10^6 $TCID_{50}$/mL) from the D.I. Ivanovsky Institute of Virology of the Russian Academy of Medical Sciences (Moscow, Russia). Stocks of influenza virus strain A/FM/1/47 (H1N1) and HSV-2 were stored at -70 ºC.

Cytotoxicity assay

MDCK and RK-13 cells were plated onto 96-well plates and incubated at 37 ºC in a humidified atmosphere with 5% CO_2. After 48 h, the monolayered cells

were incubated in the presence of a variety of concentrations (range 0.77–50 mg/mL) of the test samples. Plates were incubated for 5 days under the same conditions. The cytopathic effect in the cell monolayer was monitored daily by the cells' morphology. Maximum tolerated concentrations (i.e., the maximal non-toxic concentrations) were determined by evaluating the cytopathic effect.

Antiviral assay

The effects of the studied mushroom mycelium extracts on the process of virus multiplication were investigated in MDCK cells (for influenza virus A/FM/1/47 [H1N1]) and RK-13 cells (for herpes virus type 2). Both cell cultures were pretreated with dilutions of the clarified extract (range 0.77–50 mg/mL) for 30–60 min at 37 ºC and then infected with the viruses. The cells infected with virus-containing fluid were incubated at 37 ºC for 3 days. The infectious titers of viruses, presence of virus-specific antigens, and hemagglutinin levels were assayed in culture liquid. The infectious titer was evaluated using a series of 10-fold dilutions of virus-containing culture liquid. The half maximal effective concentration (EC_{50}) inhibiting viral reproduction was calculated.

Data analysis

All experiments were confirmed in three independent replicates. The antiviral activity of the mycelium was determined as the reduction factor ($\log_{10}$) of the viral titer by comparison with untreated controls. The standard deviation in the reduction of virus titer was about 0.5 $\log_{10}$. Mycelium was defined as active if the viral yield decreased by $\geq 2 \log_{10}$ at the maximum tolerated concentration.

RESULTS

As the first step in screening antiviral activity, the cytotoxicity of various concentrations of mycelial extracts was evaluated using an inhibition assay in MDCK and RK-13 cell plaques (with similar cytopathic effect). A maximum tolerated concentration of 25.0 mg/mL was determined for four species (*A. aurea*, *F. fomentarius*, *F. velutipes*, and *T. versicolor*), while the extracts of the other species, particularly two edible mushrooms (*P. eryngii* and *L. edodes*), were more toxic (Table 1).

The investigated mycelia had different potential antiviral activities against influenza virus strain A/FM/1/47 (H1N1), with inhibition of infectious titers ranging from 2.0 to 6.0 lg ID_{50}. Antiviral activity according to the inhibition of infectious titer increased in the following order: *A. aurea* = *F. fomentarius* > *P. ostreatus* = *L. shimeji* = *L. edodes* > *P. eryngii* = *F. velutipes* = *G. lucidum* > *S. commune* > *T. versicolor*. *G. lucidum* and *T. versicolor* generated the strongest antiviral effects, with *T. versicolor* showing the highest activity (Table 2). The anti-influenza activities of *P. eryngii*, *L. shimeji*, *F. velutipes*, and *A. aurea* have not previously been presented in the literature.

Only four of the 10 studied species demonstrated activity against HSV-2: mycelial extracts of *P. ostreatus*, *F. fomentarius*, *A. aurea* and *T. versicolor* significantly inhibited HSV-2 replication in RK-13 cells (Table 3). The highest therapeutic indices (selectivity indices) were identified for *A. aurea* and *T. versicolor*, at 161.29 and 324.67, respectively. This study is the first to demonstrate the activity of *A. aurea* mycelium against HSV-2.

DISCUSSION

Recently, the search for natural substances as raw materials for the pharmaceutical industry has revived interest in medicinal and edible mushrooms. While most studies have isolated therapeutically active substances from the fruiting bodies, the use of mycelium makes it possible to obtain products of consistent quality more quickly and at lower cost. Data regarding the absence of toxicity of various products have been obtained from cultivation, mainly on synthetic or semisynthetic substrates. The mycelium toxicity (non-critical) obtained in our studies (Table 1) can be related to the natural substrate (its toxicity), has been used by us.

The influenza and herpes viruses have been a particular focus of research. The antiviral activity of mushroom preparations has been evaluated by researchers using different indicators, including the index of neutralization (Amoros M, et al., 1997; Ibragimova Zh D, et al., 2012; Teplyakova T V, et al., 2012; Filippova E I, et al., 2012; Kostina N E, et al., 2013) and the therapeutic index (Eo S-K, et al., 1999; Oh K-W, et al., 2000; Liu J, et al., 2004; Gu C-Q, et al., 2007; Cardoso FTGS, et al., 2011). In the current study, we have shown that the magnitude of the neutralization index is not directly proportional to the magnitude of the therapeutic index (Table 2 to 4). This is to be expected, since the therapeutic index is the ratio of the minimal effective dose of a chemotherapeutic agent to the maximal tolerated dose, and this ratio can be very small (even if the neutralization index is large). So, water- and methanol-soluble substances isolated from the carpophores of *G. lucidum* have shown antiviral activity against influenza A virus A/Equine/2/Miami/1/63 strain, but the therapeutic index was zero (Eo S-K, et al., 1999). In contrast, the current study found a high therapeutic index value (80.5) for *G. lucidum* mycelial activity against influenza virus strain A/FM/1/47 (H1N1). According to our data, *F. fomentarius* mycelium shows similar activity (Table 4) to preparations from

Table 1. Cytotoxicity of mycelial extracts from of MDCK and RK-13 cells

Sample Concentration (mg/mL)	Toxic dose of sample (mg/mL) MDCK cells							Toxic dose of sample (mg/mL) RK-13 cells						
	50	25	12.5	6.2	3.1	1.55	0.77	50	25	12.5	6.2	3.1	1.55	0.77
Substrate*	10/10	0/10	0/10	0/10	0/10	0/10	0/10	10/10	0/10	0/10	0/10	0/10	0/10	0/10
F. fomentarius	10/10	0/10	0/10	0/10	0/10	0/10	0/10	10/10	0/10	0/10	0/10	0/10	0/10	0/10
F. velutipes	10/10	0/10	0/10	0/10	0/10	0/10	0/10	10/10	0/10	0/10	0/10	0/10	0/10	0/10
A. aurea	10/10	0/10	0/10	0/10	0/10	0/10	0/10	10/10	0/10	0/10	0/10	0/10	0/10	0/10
T. versicolor	10/10	0/10	0/10	0/10	0/10	0/10	0/10	10/10	0/10	0/10	0/10	0/10	0/10	0/10
P. ostreatus	10/10	10/10	0/10	0/10	0/10	0/10	0/10	10/10	10/10	0/10	0/10	0/10	0/10	0/10
S. commune	10/10	10/10	0/10	0/10	0/10	0/10	0/10	10/10	10/10	0/10	0/10	0/10	0/10	0/10
G. lucidum	10/10	10/10	10/10	0/10	0/10	0/10	0/10	10/10	10/10	10/10	0/10	0/10	0/10	0/10
L. shimeji	10/10	10/10	10/10	10/10	0/10	0/10	0/10	10/10	10/10	10/10	10/10	0/10	0/10	0/10
P. eryngii	10/10	10/10	10/10	10/10	10/10	0/10	0/10	10/10	10/10	10/10	10/10	10/10	0/10	0/10
L. edodes	10/10	10/10	10/10	10/10	10/10	0/10	0/10	10/10	10/10	10/10	10/10	10/10	0/10	0/10

0/10: no cytopathic effect; 10/10: cytopathic effect (complete destruction of monolayer cells). *: 60 g amaranth in 1 L distilled water(the liquid culture medium). All experiments were confirmed in three independent replicates.

Table 2. Antiviral activity of samples in MDCK cells infected with influenza virus strain A/FM/1/47 (H1N1)

Sample	MTC (mg/mL)	EC_{50} (mg/mL)	Therapeutic index (MTC/EC_{50})
P. eryngii	1.55	5	0
L. shimeji	3.1	0.62	5.0
P. ostreatus	12.5	2.5	6.0
S. commune	12.5	0.62	20.16
L. edodes	1.55	0.077	20.12
F. velutipes	25	1.25	20.0
F. fomentarius	25	0.62	40.32
A. aurea	25	0.62	40.32
G. lucidum	0.2	0.077	80.5
T. versicolor	25	0.077	324.67

EC_{50}: half maximal effective concentration; MTC: maximum tolerated concentration. All experiments were confirmed in three independent replicates.

Table 3. Antiviral activity of samples in RK-13 cells infected with herpes simplex virus type 2, strain BH

Sample	MTC (mg/mL)	EC_{50} (mg/mL)	Therapeutic index (MTC/EC_{50})
S. commune	12.5	0	0
F. velutipes	25	0	0
P. eryngii	1.55	0	0
L. shimeji	3.1	0	0
L. edodes	1.55	0	0
G. lucidum	6.2	0	0
F. fomentarius	25	0.62	40.32
P. ostreatus	12.5	0.155	80.64
A. aurea	25	0.155	161.29
T. versicolor	25	0.077	324.67

EC_{50}: half maximal effective concentration; MTC: maximum tolerated concentration. All experiments were confirmed in three independent replicates.

F. fomentarius 11-72, which has been reported to inhibit influenza virus A/Aichi/2/68 in MDCK cell culture at 2.4 lg (Ibragimova Zh D, et al., 2012). The fruiting bodies of *T. versicolor* have been reported to slightly inhibit influenza virus A/Chicken/Kurgan/05/2005 (H5N1) *in vivo* (Ibragimova Zh D, et al., 2012). Our results (Table 4) for *T. versicolor* 353 mycelium were significantly higher (6.0 lg) than those reported in similar studies: water extracts of *T. versicolor* 2263 mycelium have been reported to repress viruses A/Chicken/Kurgan/05/2005 (H5N1) (2.5 lg) and A/Aichi/2/68 (H3N2) (0.5 lg) on MDCK cells (Teplyakova TV, et al., 2012). In the current study, *L. shimeji*, *L. edodes* and *P. ostreatus* mycelia showed neutralization indices for influenza virus strain A/FM/1/47 (H1N1) that were similar to the values obtained by Filippova EI, et al. (2012) for fruiting bodies of *Ganoderma applanatum*, *Inonotus* obliquus and *Laetiporus sulphureus* against pandemic influenza virus A/Moscow/226/2009 (H1N1).

Our data demonstrating sufficiently high antiherpetic activity of *P. ostreatus* mycelium (neutralization index of 2.5 lg and therapeutic index of 80.64; Table 3 and 4) are in contrast to those indicating an absence of antiherpetic activity for *P. ostreatus* fruiting bodies in cell culture (Amoros M, et al., 1997). Our data also show significant antiherpetic activity for *F. fomentarius* mycelium

Table 4. Antiviral activity of samples

Sample	virus H1N1, strain A/FM/1/47		virus HSV-2, strain BH	
	Infectivity of the virus (VAF titer) in MDCK cells (ID_{50} in lgTCID$_{50}$/mL)	Neutralization index (ID_{50} control – ID_{50}exp.), lg	Infectivity of the virus (VAF titer) in RK-13 cells (ID_{50} in lgTCID$_{50}$/mL)	Neutralization index (ID_{50}control – ID_{50}exp.), lg
P. eryngii	2.0	4.0	6.0	0
L. shimeji	3.0	3.0	6.0	0
P. ostreatus	3.0	3.0	3.5	2.5
S. commune	1.0	5.0	6.0	0
L. edodes	3.0	3.0	6.0	0
F. velutipes	2.0	4.0	6.0	0
F. fomentarius	4.0	2.0	3.0	3.0
A. aurea	4,0	2.0	4.0	2.0
G. lucidum	2.0	4.0	6.0	0
T. versicolor	0	6.0	0	6.0
Substrate[a]	6.0	0	6.0	0
Control (virus)	6.0[b]	–	6.0[c]	–

Note: HSV-2, herpes simplex virus type 2; TCID$_{50}$, median tissue culture infective dose. The standard deviation for the reduction of virus titer was approximately 0.5 log$_{10}$. a: 60 g amaranth in 1 L distilled water (the liquid culture medium). b: virus H1N1, strain A/FM/1/47; c: virus HSV-2, strain BH. All experiments were confirmed in three independent replicates.

(neutralization index 3.0 lg and therapeutic index 40.32; Table 3 and 4), in contrast to data showing the absence of such activity in fruiting bodies of this fungus in cell culture (Kostina N E, et al., 2013). Conversely, *G. lucidum* mycelium did not show antiherpetic action in our studies, unlike the results of other researchers who used polysaccharide–protein complexes isolated from *G. lucidum* fruiting bodies and mycelia, including APBP (activity against HSV-2 strain 233) (Oh KW, et al., 2000) and proteoglycan (activity against HSV-2 G strain ATCC VR-734) on Vero cells (Liu J. et al, 2004.). The selectivity index values for *P. ostreatus*, *A. aurea*, and *T. versicolor* mycelia were significantly higher in our studies than in similar experiments with *G. lucidum* mycelium against HSV-2 (Oh KW, et al., 2000; Liu J, et al., 2004). Our results indicate antiherpetic activity of *T. versicolor* mycelium, in contrast to other investigations with fruit bodies of this species (Amoros M, et al., 1997; Kostina NE, et al., 2013). Moreover, in the current study, *T. versicolor* showed the highest therapeutic index (324.67) among the studied fungi. A clinical trial in which a food additive (biomass) of *T. versicolor* reduced the frequency and even stopped outbreaks of HSV-2 in pregnant patients (French A, 2007) confirms the high antiherpetic activity of this fungus.

It should be noted that the effective doses of *P. ostreatus* and *A. aurea* mycelium for HSV-2 neutralization were significantly lower than those for influenza virus A, with 13-fold higher antiherpetic activity than anti-influenza activity for *P. ostreatus* and fourfold higher antiherpetic activity for *A. aurea*. In contrast, the therapeutic indices of *F. fomentarius* and *T. versicolor* were identical for both viruses. Such differences may be caused by mushroom species specificity, variability in the biologically active substances, and different mechanisms of antiviral activity at the interaction between the mycelia and the virus. The high efficacy of viral replication inhibition might hint that the inhibitory activity of the tested substances occurs late in viral replication via impairment of viral protein synthesis.

This study of the antiviral activity of the mycelia of 10 mushroom species suggests that *A. aurea*, *F. velutipes*, *F. fomentarius*, *G. lucidum*, *L. edodes*, *L. shimeji*, *P. eryngii*, *P. ostreatus*, *S. commune*, and *T. versicolor* have antiviral activity against influenza virus A/FM/1/47 (H1N1), while *A. aurea, F. fomentarius, G. lucidum,* and *T. versicolor* have antiviral activity against HSV-2, strain BH. For some of the investigated species, this is the first report of anti-influenza (*P. eryngii, L. shimeji, F. velutipes, A. aurea*) and antiherpetic (*A. aurea*) effects. To the best of our knowledge, there have been no previous reports on the potential medicinal properties of *A. aurea*.

The wood-decaying medicinal Basidiomycete *T. versicolor* showed the highest therapeutic index (324.67 for both viruses). Therefore, *T. versicolor* 353

and its biologically active substances may be promising source materials for the pharmaceutical industry as anti-influenza and antiherpetic agents with low toxicity. The use of products obtained in the biotechnological processing of agricultural waste (in this case, amaranth flour after CO_2 extraction) and its conversion by fungi is one of the first steps in this direction of investigations, which deserves to be further explored. Further investigations will be needed to determine the most effective solvent for extracting biologically active mycelial substances; to study the qualitative and quantitative composition, antiviral activity, and mechanisms of antiviral activity of the extracted mycelial substances; and to confirm the effectiveness of *T. versicolor* mycelium *in vivo*.

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COMPLIANCE WITH ETHICS GUIDELINES

All the authors declare that they have no competing interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

AUTHOR CONTRIBUTIONS

Tetiana Krupodorova and Victor Barshteyn conceived the study, cultivated mushrooms, obtained mycelia, participated in data analysis, and wrote the manuscript. Svetlana Rybalko carried out the antiviral assay in cell cultures and participated in data analysis.

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