Rustmicin, a Potent Antifungal Agent, Inhibits Sphingolipid Synthesis at Inositol Phosphoceramide Synthase*

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Rustmicin is a 14-membered macrolide previously identified as an inhibitor of plant pathogenic fungi by a mechanism that was not defined. We discovered that rustmicin inhibits inositol phosphoceramide synthase, resulting in the accumulation of ceramide and the loss of all of the complex sphingolipids. Rustmicin has potent fungicidal activity against clinically important human pathogens that is correlated with its sphingolipid inhibition. It is especially potent against Cryptococcus neoformans, where it inhibits growth and sphingolipid synthesis at concentrations <1 ng/ml and inhibits the enzyme with an IC50 of 70 pM. This inhibition of the membrane-bound enzyme is reversible; moreover, rustmicin is nearly equipotent against the solubilized enzyme. Rustmicin was efficacious in a mouse model for cryptococcosis, but it was less active than predicted from its in vitro potency against this pathogen. Stability and drug efflux were identified as two factors limiting rustmicin’s activity. In the presence of serum, rustmicin rapidly epimerizes at the C-2 position and is converted to a γ-lactone, a product that is devoid of activity. Rustmicin was also found to be a remarkably good substrate for the Saccharomyces cerevisiae multidrug efflux pump encoded by PDR5.

Cryptococcus neoformans is a basidiomycetous fungus that infects immunocompromised patients, initiating in the lungs and migrating to the central nervous system, where it results in meningocencephalitis. Over 80% of cryptococcosis cases are found in advanced stage human immunodeficiency virus patients, and C. neoformans is one of the most common opportunistic infections in this population. In recent years, the incidence has declined (1, 2). The decline has been attributed to the widespread use of fluconazole, a fungistatic agent that is effective but requires lifelong suppressive therapy to prevent relapse in AIDS patients. Fluconazole has also been used successfully to treat fungal infections caused by Candida albicans and other Candida species, but a worrisome development is the emergence of species that are intrinsically resistant to azoles, such as Candida glabrata and Candida krusei, as well as the appearance of a variety of Candida isolates that have acquired resistance. Aspergillus and Fusarium species are mycelial fungi that are also relatively resistant to fluconazole; these pathogens are particularly aggressive and the incidence of mortality from aspergillosis is very high (2). Increased use of prophylactic antifungal therapy will probably exacerbate the resistance problem; thus, new treatments are urgently needed.

We have been investigating the sphingolipid pathway as a novel target for antifungal therapy. Serine palmitoyltransferase, the first committed enzyme of sphingolipid synthesis in mammalian and fungal cells, condenses serine and palmitoyl-CoA to form the long chain sphingoid base, ketodihydrosphingosine. Several structurally diverse natural product inhibitors of this enzyme with antifungal activity have been discovered including sphingofungins (3, 4), myriocin (5), lipoxamycins (6), and viridiofungins (7). Ketodihydrosphingosine is reduced to dihydrosphingosine and can be further modified to phytosphingosine in fungi and sphingosine in mammals. Desaturation of the sphingoid base to sphingosine takes place after condensation with a fatty acyl-CoA to form ceramide (8), whereas phytosphingosine synthesis probably occurs before acylation, based on the accumulation of phytosphingosine that occurs when ceramide synthesis is inhibited (9, 10). Two classes of natural product inhibitors of ceramide synthase have been described: fumonisins (11) and australafungin (9). The fumonisins are mycotoxins isolated for their tumor promoting activity (12); they cause profound effects on mammalian cells that have been attributed to inhibition of ceramide synthesis and the concomitant accumulation of sphingoid bases (13). Fumonisin B1 has relatively poor whole cell activity against fungi, although it inhibits C. albicans ceramide synthase in vitro. In contrast, australafungin has potent, broad spectrum antifungal activity (9), but little is known about its proliferative or toxic effect on mammalian cells, although it is known to inhibit ceramide synthesis in HepG2 cells (14).

Most of the ceramide and sphingoid bases in cells are not free but instead are found in complex lipids. In fungi, phosphoinositol is transferred from glycerophosphatidylinositol to the C-1 hydroxyl of ceramide to make inositol phosphoceramide (IPC). IPC is further modified by the addition of mannose and a second inositol phosphate group to make mannosyl inositol phosphoceramide and mannosyl diinositol diphosphoceramide (15). In mammalian cells, the only phosphosphingolipid is sphingomyelin, which contains phosphocholine at the C-1 hydroxyl of ceramide. There is, however, an enormous class of glycosylated sphingolipids containing a variety of complex modifications of the carbohydrate moiety. Some fungi, including pathogenic species of Aspergillus, have been reported to make glycosphingolipids (16), but the major sphingolipids of C. albicans and C. neoformans have the phosphoinositol moiety

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1 The abbreviations used are: IPC, inositol phosphoceramide; HPLC, high pressure liquid chromatography; PI, phosphatidylinositol; YNBD, yeast nitrogen base with glucose; MIC, minimum inhibitory concentration; CFU, colony-forming units.
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(15, 17). Recently, two antifungal agents that inhibit inositol phosphoceramide synthase were described, aureobasidin A1 (18) and khafrewfungin (14). Khafrewfungin was found to be selective at inhibiting sphingolipid synthesis in fungi, whereas compounds that affect earlier steps in the pathway also inhibit mammalian enzymes.

During the course of our studies on inhibitors of sphingolipid synthesis, we discovered that rustmicin selectively inhibits fungal sphingolipid synthesis. Rustmicin is a macrolide antifungal agent that was isolated over 10 years ago by two independent groups. It was isolated from fermentations of M. cromonospora chalcea and named rustmicin for its activity against wheat stem rust fungus (Puccinia graminis) (19). Almost simultaneously, the same structural compound was reported as galbonolide A from Streptomyces galbus culture broths, with potent activity against Botrytis cinerea and several other phytopathogens (20, 21). Mode of action studies indicated that rustmicin did not destabilize the membrane or inhibit the synthesis of chitin, DNA, or RNA, but the mechanism of fungal growth inhibition was not determined (22). We have discovered that rustmicin has extraordinarily potent antifungal activity against several human pathogens, especially C. neoformans. Moreover, its antifungal activity is due to inhibition of sphingolipid synthesis at the IPC synthase, where rustmicin demonstrates inhibition at picomolar levels. The sphingolipid inhibitory activity and the in vitro and in vivo antifungal activity of rustmicin are presented in this paper.

**EXPERIMENTAL PROCEDURES**

**Strains, Inhibitors, and Reagents—**Saccharomyces cerevisiae W303–1A (MATa ade2–1 can1–100 his3–11, 15 leu2–3, 112 trp1–1 ura3–1) was provided by R. Rothstein (23). C. albicans MY1055, C. neoformans MY2062, and other fungal pathogens were obtained from the Merck Culture Collection (Rahway, NJ). For in vitro assays, C. neoformans strain Cap64 (ATCC52616) was used. The in vitro antifungal and fungicidal assays were performed using the drug and the fungus at concentrations of 0.1 and 1.0 μg/ml, respectively.

**Sphingolipid and IPC Synthesis—**Sphingolipid synthesis and IPC synthesis were measured as described previously (14). Briefly, fungal cells were labeled with [3H]inositol in microtiter plates, and aliquots of 0.1 ml were added to the plates. The plates were incubated at 30°C for 48 h. After 48 h growth at 31°C, the cells were harvested, and the inositol biosynthesis inhibitors were removed periodically, diluted, and plated onto YNBD agar. Colony-forming units (CFU) were enumerated after 48 h growth at 30°C.

**In Vivo Cryptococcosis Assay—**DBA2N mice were infected by intravenous inoculation with approximately one LD₅₀ of C. neoformans MY2061 (1 × 10⁶ cells/mouse). Treatment was initiated within 15 min after challenge, and the mice were treated for a total of 4 days. At 7 days after challenge, brains and spleens were harvested, and CFU were determined as described (25). Rustmicin was solubilized in 5% ethanol, 20% polyethylene glycol, and dosed intraperitoneally twice daily. Amphotericin B (0.31 mg/kg) was administered intraperitoneally once daily. Five mice per treatment group were tested. All procedures were performed in accordance with the highest standards for the humane handling, care, and treatment of research animals and were approved by the Merck Institutional Animal Care and Use Committee.

**RESULTS—**Rustmicin was diabetogenic. Rustmicin inhibited inositol incorporation into sphingolipids in vitro and in vivo antifungal activity of rustmicin are presented in the paper.

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2 Y. S. Tang, unpublished data.
When tested in in vitro enzyme assays, rustmicin did not inhibit two enzymes early in the sphingolipid biosynthetic pathway, serine palmitoyltransferase and ceramide synthase, but was a very potent inhibitor of IPC synthase. Labeling studies with sphingolipid precursors confirmed that IPC synthase was the step in the sphingolipid pathway inhibited by rustmicin, since the inhibitor caused the accumulation of hydroxyceramide (data not shown), the same lipid intermediate that was previously seen upon khafrefungin treatment (14).

**IPC Synthase Inhibition by Rustmicin**—The enzyme was assayed in microsomal membrane preparations by measuring incorporation of trace \[^{3}H\]ceramide into \[^{3}H\]IPC, as described previously (14). Cholate and exogenous PI were employed in the assay because they markedly enhanced the reaction, which was particularly slow for *C. neoformans*. Omission of these components had little influence on the potency of inhibitors. As shown in the titrations of Fig. 3A, rustmicin has an IC\textsubscript{50} of 70 nM.
strains, which are values similar to the compound’s sphingo-

Rustmicin concentration for 50% inhibition of sphingolipid synthesis and in vitro IPC synthase

| Organism          | IPC synthase IC₅₀ ± S.D. | Sphingolipid synthesis IC₅₀ nm |
|-------------------|--------------------------|-----------------------------|
| C. neoformans     | 0.07 ± 0.01              | 6.5                         |
| C. albicans       | 3.80 ± 0.34              | 65.0                        |
| S. cerevisiae     | 19.80 ± 2.12             | 92.0                        |

TABLE II shows that rustmicin was extremely potent against various fungi, primarily plant pathogens, and found to have MIC values of 0.1 µg/ml to >1 mg/ml (21, 22). A few human pathogens were tested, including C. albicans, which had an MIC of 3 µg/ml, and Aspergillus fumigatus, which was insensitive to rustmicin. The advent of AIDS and the emergence of azole-resistant fungi have changed the clinical spectrum of organisms in recent years, so we evaluated rustmicin for inhibitory activity against a current panel of human pathogenic fungi.

Table II shows that rustmicin was extremely potent against C. neoformans, with MIC values of 0.1–1 ng/ml against various strains, which are values similar to the compound’s sphingo-

with a membrane active agent such as amphotericin B that causes rapid cell leakage (Fig. 4). Loss in viability with rustmicin treatment was only seen in actively growing cultures (data not shown).

In Vivo Antifungal Activity—The extraordinarily potent inhibition of C. neoformans led us to evaluate the efficacy of rustmicin in a mouse model for cryptococcosis. Fig. 5 shows that rustmicin treatment produced a dose-dependent reduction in colony-forming units isolated from spleen and brain tissue of mice infected with C. neoformans. The ED₉₀ levels were calculated to be 29 mg/kg for both tissues. In another experiment in which compounds were suspended in olive oil to overcome solubility limitations and dosed at 100 mg/kg, rustmicin gave 100% sterilization of brains and spleen. Although encouraging, the level of in vivo efficacy is far less than expected from the in vitro susceptibility of this organism to rustmicin. Amphotericin B, which has an MIC against C. neoformans that is 1000-fold higher than rustmicin, was fully effective in the mouse cryptococcosis model at 0.31 mg/kg (Fig. 5). We therefore undertook an analysis of some of the factors limiting the in vitro activity of rustmicin.

Stability of Rustmicin—When originally isolated, fermentation extracts containing galbonolide A were found to be unstable, and several chemical degradative pathways were eluci-
dated (22). The enol ether moiety was susceptible to acid treatment, whereas alkaline reagents caused epimerization at the C-2 position and conversion of the macrolactone to a γ-lactone (see Fig. 1). Using antifungal activity against C. albicans as a bioassay and HPLC analysis to monitor chemical stability, we tested whether degradation of rustmicin was a limitation for in vivo efficacy. In agreement with the previous study, we found that rustmicin decomposed within minutes in alkaline or acidic buffer, with loss of all bioactivity (Fig. 6, A and B). The half-life of the compound at neutral pH was approximately 80 min, and the best stability was found at pH 5.5.

The effect of 50% whole mouse serum on rustmicin stability and activity was tested to better represent the in vivo condition. In the presence of serum, the rate of inactivation was accelerated (Fig. 7A), and two degradation products were found (Fig. 7B). The C-2 epimer (L-760,262) appeared rapidly and then was also degraded, while the product that accumulated in direct proportion to rustmicin disappearance was the translactonized compound (L-770,715). The same products were formed in the absence of serum, indicating that these are due to the chemical instability of rustmicin. Analysis of the bioactivity of the degradation products showed that epimerization resulted in a 60–80-fold loss in potency of inhibiting sphingolipid synthesis in C. neoformans and C. albicans, while the γ-lactone was completely inactive (Table III). Thus, poor in vivo efficacy in animals is probably due to a great extent to the rapid conversion of rustmicin to the inactive γ-lactone.

Resistance to Rustmicin—Multidrug efflux pumps eliminate structurally diverse inhibitors from mammalian and fungal cells, and many gene products that participate in drug transport or regulation of the transporters have been identified in S. cerevisiae (28). We used two S. cerevisiae mutants, pdr5:Tn5 and pdr1–3, to evaluate the role of multidrug resistance in rustmicin’s potency. Pdr5p is one of the major ATP binding cassette efflux pumps, and disruption causes hypersensitivity to many antifungals, while pdr1–3 is a mutation in a transcriptional regulator that increases expression of PDR5 and other genes and results in resistance to many inhibitors (24, 28). In MIC tests with cycloheximide, a substrate for Pdr5p, the pdr5 null mutant was 8-fold more sensitive, and pdr1–3 was 2-fold more resistant than the control strain (Fig. 8A). Several other inhibitors that we tested, including fluconazole, mevinolin, anisomycin, and rustmicin, also showed the same profile of sensitivity and resistance, but rustmicin was unique in its extreme differential in activity; pdr5:Tn5 was 256-fold more sensitive and pdr1–3 was 8–16-fold more resistant than the wild-type strain (Fig. 8B). The increased susceptibility of the pdr5 null strain brings the potency of rustmicin as an antifungal agent (MIC = 8 ng/ml) down to the level of IPC synthase inhibition.
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Table III

| Compound     | C. neoformans IC50 | C. albicans IC50 |
|--------------|---------------------|------------------|
| Rustmicin    | 0.0002              | 0.025            |
| L-760,262    | 0.013               | 2.0              |
| L-770,715    | >32                 | >32              |

![Graph A](image.png)

**Fig. 8. Antifungal activity in multidrug efflux mutants of S. cerevisiae.** Growth inhibition in a microbroth dilution assay by cycloheximide (A) or rustmicin (B) was measured in mutants pdr1–3 (○) and pdr5-Tn5 (■), and compared with wild type (□). The wild-type strain shown is isogenic to pdr1–3, but similar results were obtained with W303-1A and the isogenic wild-type of pdr5-Tn5.

![Graph B](image.png)

**DISCUSSION**

Sphingolipids comprise a small but essential fraction of fungal phospholipids. Differences between mammalian and fungal sphingolipid biosynthesis, especially at later steps in the pathway, make this an attractive new target for antifungal therapy. Many natural products have been discovered as inhibitors of sphingolipid biosynthesis; these compounds, which are primarily produced by fungi, have potent, fungicidal activity against a broad spectrum of clinical pathogens (3–7, 9, 14, 29). Our studies in this area led to the rediscovery4 of an actinomycete product known as rustmicin or galbonolide A (19, 20, 22). This compound is a 14-membered macrolide, previously isolated for its antifungal activity against phytopathogens but whose mechanism of fungal growth inhibition was not determined.

**TABLE III**

Concentration of rustmicin and degradation products for 50% inhibition of sphingolipid synthesis

| Compound     | C. neoformans IC50 | C. albicans IC50 |
|--------------|---------------------|------------------|
| Rustmicin    | 0.0002              | 0.025            |
| L-760,262    | 0.013               | 2.0              |
| L-770,715    | >32                 | >32              |

4 J. M. Sigmund and C. F. Hirsch, submitted for publication.

and die. In mammalian cells, ceramide has been proposed to be a key intermediate in lipid signal transduction pathways leading to apoptosis and stress response (31–34). A similar pathway appears to operate in *S. cerevisiae* where ceramide induces G1 arrest via a ceramide-activated protein phosphatase (35) and promotes cell death in an IPC synthase mutant that responds to the addition of phytosphingosine by accumulating ceramide (18). Thus, activation of a ceramide death response may confer the observed fungicidal activity to inhibitors of IPC synthase. If so, then ceramide toxicity is a slow process, since the loss in viability required several generations to manifest itself.

Alternatively, depletion of IPC or one of its further metabolites by rustmicin may induce cell death, and arguments can be made for the involvement of complex sphingolipids in several essential processes. Glycosyl phosphatidylinositol-anchored mannoproteins constitute a major component of fungal cell walls; initially, they are synthesized with PI as the lipid moiety, but many are subsequently remodeled to IPC (36). The sphingolipid intermediate that is used for replacement has not yet been established, so it is not clear whether inhibitors of IPC synthase would block this process. Sphingolipids have also been shown to be important for the processing of glycosyl phosphatidylinositol-anchored proteins through the secretory pathway (37). Recently, another role for sphingolipids in vesicular secretion has been proposed, based on studies of suppressors of the *sec14* mutant encoding the phosphatidylinositol-phosphatidylcholine transfer protein (38). These studies point to a requirement for diacylglycerol in vesicular budding from the Golgi and indicate that the rate of sphingolipid synthesis is important in regulating diacylglycerol concentrations due to the two biosynthetic steps that generate diacylglycerol: IPC and mannosyl diinositol diphasphoceramide synthesis, both of which transfer phosphoinositol from PI to the sphingolipid backbone. Rustmicin inhibits the synthesis of all of the complex sphingolipids and would be predicted to reduce diacylglycerol production at the ER, where IPC is thought to be synthesized, and at the Golgi, where the mannose-containing sphingolipids are made (39). Yet another function for sphingolipids in vesicular secretion and/or endocytosis has been proposed, due to the presence of the very long chain fatty acids (C24 and C26 species) that are complexed with sphingoid bases to form ceramide. In yeast, most of the very long chain fatty acids are found in the sphingolipid fraction; these fatty acids have been suggested to be important for providing physical stability to highly curved membranes, thereby allowing membranes to undergo budding and fusion (40). For any of these potential roles, dilution of the existing pools of sphingolipids would be required before cell death and would be consistent with the kinetics of rustmicin’s fungicidal activity and requirement for actively growing cultures to induce cell death.

In addition to its activity in *Saccharomyces*, where the role of sphingolipids is beginning to be elucidated, rustmicin also has potent antifungal activity against clinically important human pathogens, suggesting that the requirement for sphingolipids is conserved among these fungi. *A. fumigatus* is one of the few human pathogens that is insensitive to any of the known IPC synthase inhibitors (14, 29). The reason for this resistance is not known, since inhibitors of earlier steps in the sphingolipid biosynthetic pathway do inhibit the growth of *A. fumigatus*. Khafrefungin, which is composed of an aldonic acid esterified to a linear C22 polyketide, is most potent against IPC synthase of *C. albicans* (14). Similarly, the cyclic depsipeptide compound, aureobasidin A, which has been shown to inhibit IPC synthase in *S. cerevisiae* (18), is most potent as an antifungal agent against *C. albicans* (29), presumably due to its sphingolipid inhibition.
Among the three structurally diverse IPC synthase inhibitors, rustmicin is unique in its remarkable activity against C. neoformans, where it is an exceptionally potent inhibitor of IPC synthase. Rustmicin inhibits the enzyme from C. neoformans with an IC₅₀ of 70 pm, and is a low nanomolar inhibitor of the enzymes from C. albicans and S. cerevisiae. The complexity of the system, which consists of a membrane-bound enzyme operating on membrane components, precluded determining the kinetic mechanism of inhibition, but simple wash-out experiments clearly showed that rustmicin is a reversible inhibitor. In addition, it was possible to show that potent inhibition extends to the solubilized enzyme from C. neoformans, establishing that inhibition by rustmicin is specific to the enzyme and not due to possible effects on its membrane environment. Recovery of active enzyme from the supernatant required the inclusion of PI during solubilization. We attribute this effect either to stabilization of the enzyme by binding one of its substrates, a property common to many enzymes, or to preservation of a membrane-like environment in the mixed detergent/phospholipid micelles. Solubilization of IPC synthase from S. cerevisiae did not require the addition of PI, a difference that may be due to the use of a 5-fold higher ratio of membrane-protein to detergent, in which the cellular phospholipids may obviate the need for exogenous PI, or the inclusion of glycerol during solubilization (41).

Rustmicin’s affinity for the C. neoformans enzyme contributes, but cannot fully account for, the large differential in antifungal activity between C. neoformans and C. albicans, which is 4–5 orders of magnitude. Two factors that limit rustmicin’s whole cell activity, instability and multidrug efflux pumps, may have more influence on rustmicin’s activity against C. albicans and S. cerevisiae over the extended time period of the fungal growth assays. Both of these yeasts have active plasma membrane H⁺-ATPases that rapidly acidify the media of growing cultures (42). Rustmicin is degraded within minutes at low pH (Fig. 5), and buffering the media at pH 5.5 to promote stability improved rustmicin’s anti-Saccharomyces and anti-Candida activity by 2–8-fold (data not shown). Perhaps of greater impact on whole cell activity is the activity of efflux pumps. Several multidrug efflux pumps have been identified in C. albicans and S. cerevisiae and one of the mechanisms for fluconazole resistance in C. albicans has been shown to be via drug efflux pumps (24, 28, 43). We found that disruption of PDR5 dramatically improved rustmicin’s potency against S. cerevisiae. Multidrug efflux mechanisms and plasma membrane H⁺-ATPase activity have not been characterized in C. neoformans, but rustmicin’s equal potency of growth inhibition and in vitro enzyme inhibition suggest that these factors are not important for this pathogen, at least in the laboratory. However, we believe that the conversion of rustmicin to the inactive y-lactone that occurs rapidly in serum, compromises in vivo efficacy and best accounts for the relatively weak activity that rustmicin has in reducing cryptococcal load in mice, compared with its in vitro potency.

Multidrug efflux affects the activity of many structurally and mechanistically unrelated inhibitors in yeast, and in mammalian cells is one of the major obstacles to successful chemotherapy. In S. cerevisiae, disruption of PDR5 increases sensitivity to a number of compounds (24), while mutations in PDR1 that result in transcriptional activation of several genes, including PDR5, have been found to confer resistance to over 30 compounds (28). Thus, the response we obtained to rustmicin with these mutants was not surprising, but the magnitude of the effect was far more dramatic than any of the other antifungals we examined. In mammalian cells, a special relationship between multidrug efflux and glycosphingolipid synthesis was proposed due to the observed accumulation of glucosylceramides in multidrug-resistant tumor and cancer cell lines (44) and the discovery that compounds that reverse multidrug resistance inhibit glucosylceramidase synthesis (30). It is intriguing to consider that sphingolipid synthesis in yeast may also contribute to drug resistance via the efflux pumps. For instance, the global transcriptional activation seen in PDR1 mutants may also include up-regulation of sphingolipid synthesis, and/or inhibition of sphingolipid synthesis may regulate the activity of the yeast efflux pumps. We have found that yeast cells with reduced levels of sphingolipids, either by mutation or drug treatment, are hypersensitive to many unrelated antifungals. However, drug hypersensitivity is also found in sterol synthesis mutants and has been attributed to changes in membrane fluidity and permeability (26). This explanation could account for the sphingolipid effect, and the differential activity of rustmicin against the drug-resistant strains may simply reflect affinity for PDR5. The multidrug resistance network has been well developed in yeast (28) and provides the genetic tools that, in combination with the lipid biosynthetic inhibitors and mutants, could help define the interactions between lipid synthesis and drug efflux pumps. Rustmicin, despite its in vivo limitations, is a highly potent IPC synthase inhibitor and will be an important tool to study the role of the inositol sphingolipids in yeast and fungal cell physiology.

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