Tunicates: A model organism to investigate the effects of associated-microbiota on the production of pharmaceuticals

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Ascidians are marine invertebrates closely related to vertebrates. These animals have been studied to address complex processes, including evolution of the immune system and developmental biology. As holobionts, housing millions of bacteria in a close relationship that drives adaptive fitness to environmental conditions, ascidians are successful invaders and dominant components of the benthic communities. Further, tunicates and their associated microbiota are recognized as producers of chemical structures with pharmacological potential, and over 1000 such molecules have been described so far. This review covers aspects of ascidian biology that make them promising model organisms in various fields and important for drug discovery.

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Introduction

Tunicates (or urochordates) are marine invertebrate filter feeders belonging to the phylum Chordata, which also includes cephalochordates and vertebrates. The presence of a notochord is the main shared character, or synapomorphy, that groups these organisms into the same phylum. Ascidians (class Ascidiacea), popularly known as sea squirts, are the most speciose class of tunicates, with about 3000 species described so far. They can be found throughout marine

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environments, from shallow to deep waters [1]. Tunicates are currently considered the closest relatives of vertebrates [2,3], suggesting that they are a good model organism for developmental and evolutionary studies, particularly regarding the immune system. As filter-feeders, ascidians are highly exposed to environmental factors such as pathogens. Therefore, their immune system should be complex and well-structured. The tunic, a living tissue containing a complex structure based on a cellulosic matrix, is considered the first barrier against pathogens and predators. The oral siphon and pharynx function as the main immune organs, and can detect foreign material and activate the immune system [4].

As both shelter and source of nutrients, these organisms can provide an attractive environment for the development of a diverse and abundant microbiota. The increasing attention to the microorganisms associated with animal hosts, as well progress in our knowledge of their roles and mutual interactions, led to the establishment of the holobiont paradigm [5]. Within marine animals, ascidians have been considered and studied as a holobiont system, in which the invertebrate harbors a diverse assemblage of microorganisms. The microbiota of ascidians is still poorly known, as most of these microorganisms cannot be detected through typical culture methods. Recent advances in metagenomic techniques have allowed deeper investigation of the associated-microbiota in holobionts. Nonetheless, the relationship between ascidians and their microbiota has not yet been thoroughly investigated, and their effects on the host immune system are mostly unknown.

Along with sponges and bryozoans, ascidians can avoid fouling and predation by producing secondary metabolites. They are therefore considered a source of new chemical structures with remarkable pharmacological potential, with over 1000 such molecules described so far [6,7]. Trabectedin and plitidepsin are the only two compounds already ap-proved for clinical use in cancer therapy, but there are others in clinical trials.

More recently, the production of some of the metabolites described from ascidians was attributed to associated bacteria embedded in the tunic matrix, in a syntrophic association with the tunicate [8,9]. In general, symbiotic bacteria produce compounds that can be used by their host, especially in some kind of defensive function, including combating pathogens, deterring predators, and for photoprotection. Fig. 1 shows (a) pictures of some ascidians our research group has studied; (b) the position of ascidians (Ascidiacea) in the phylogeny of chordates; and (c) a representative scheme of ascidian body as a host of a microbial community, and moreover, how this holobiont system can be translated into complex chemical structures. However, several questions regarding ascidian-associated microbiota still remain unanswered. Therefore, this review addresses the tunicates as model organisms from different points of view, including (i) a brief overview of the evolution and development of the immune system in a microbial context; (ii) consideration of the relationship between this invertebrate and its associated microbiota, focusing on functional aspects; and, finally (iii) the resulting chemical diversity and pharmaceutical potential of such molecules.

Ascidians as a model organism for investigation of the evolution of the immune system

As the closest living relatives of vertebrates among the chordates [2,11], tunicates are a good example of simplification of the body as an adaptation to their sessile filter-feeding habits. The group has many peculiarities that puzzled naturalists for a long time. For instance, ascidians lose most of their chordate characters as adults, since their bodies are encapsulated by a tissue with a unique cellulose matrix and vacuoles filled with sulfuric acid. In addition, many species can accumulate
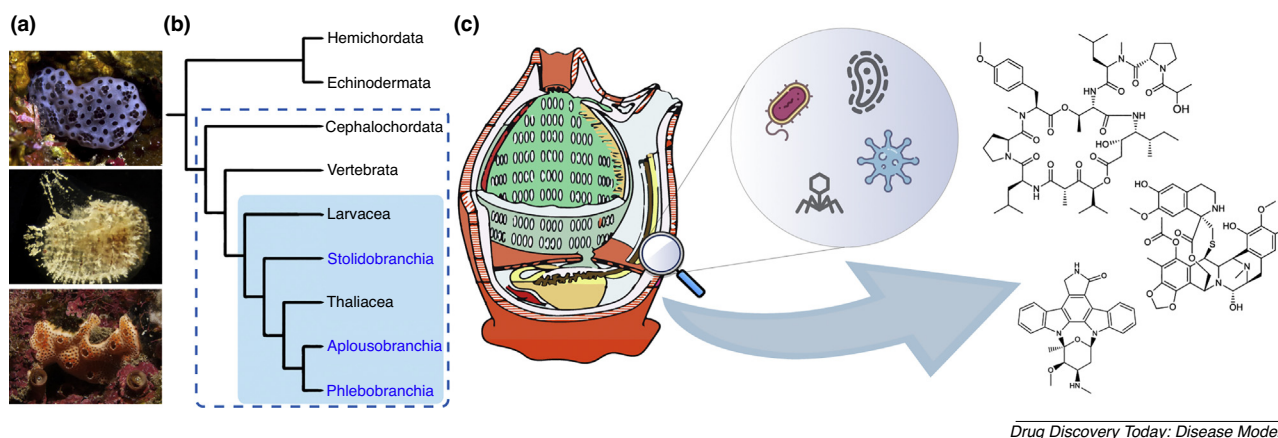
Fig. 1. (a) Pictures of ascidians, from top to bottom: *Eudistoma versicolor* (colonial), *Corella minuta* (solitary) and *Didemnum ligulum* (colonial); (b) Proposed phylogeny of chordates according to Kocot, et al. [10], showing Chodata in the doted box and Tunicata in the blue box. Ascidiacea taxa have blue labels. (c) Solitary ascidian and its body divisions, representing a host for a microbial community. Ascidians, as well as their associated microbiomes, are recognized as a rich source of compounds with remarkable pharmacological potential.

metals such as vanadium, chromium and molybdenum in their hemolymph cells [12].

The ascidian body division and organ structures vary according to the taxon concerned. The class was typically divided into three orders based mainly on the structure of the pharynx (Aplousobranchia, Phlebobranchia and Stolidobranchia). This scheme has been partially challenged by more recent phylogenetic reconstructions with larger taxon sampling and full genome data, proposing Thaliacea as a clade nested within ascidians [10]. Ascidian species may also be solitary or form colonies, and this latter form of organization seems to have evolved multiple times within the Ascidiacea.

As a sessile benthic animal, ascidians soon became a model for studying embryogenesis, as some species are easily reared in the laboratory and have interesting biological traits [13]. A model organism must have a set of features that makes them suitable for the study of complex processes. In this case, ascidians are particularly appropriate, not only due to their phylogenetic position, but also because of their simplicity. Hence, the solitary species *Ciona intestinalis* and *Ciona robusta* were chosen by many researchers as models for developmental studies. Their zygotes and blastomeres are fairly large and easily handled [14]. In addition, the complete process of embryogenesis is fast, occurring in about 18 h from the zygote to a fully developed larvae [15].

The interest in ascidians as biological models spans other aspects as well, such as immunology and regenerative biology. Regarding regeneration processes studies, *Ciona* spp. have been assessed for over a century [16]. *Botryllus schlosseri* and other colonial Styelidae have been also studied, and different regeneration processes have been shown for these animals, including whole body regeneration promoted by stem cells [17,18].

The immune system of ascidians has been extensively investigated over the last few decades. Despite their close relationship with vertebrates, tunicates rely only on innate immunity, lacking any long-term immunological memory [4]. In this context, *Ciona* spp. have also been frequently used as a model for immunological studies. Another species, *Halocynthia roretzi*, has been further indicated for investigations with this focus particularly due to their large bodies and considerable hemolymph volume, being farmed in Japan and Korea as a seafood delicacy. Other relevant models for these types of studies are botryllids, especially *Botrylloides violaceus* and *B. schlosseri*. The latter is among the only colonial ascidians reared in laboratory for scientific studies. In fact, the mechanisms of allorecognition was particularly well-studied in botryllid ascidians [19].

The various tissues and organs of the ascidians are differently challenged in terms of the immune response. The outer layer of protection is conferred by the tunic, which remains in direct contact to the environment, but in a more passive manner [4]. The oral syphon and pharynx, however, are under extreme immune pressure due to the continuous influx of seawater for alimentary and respiratory purposes. The perforated pharynx is generally the largest organ in an ascidian, being spacious enough to host not only microorganisms, but also large animals such as shrimps, worms and copepods. Ascidians have a varied immune repertoire, including immunocytes, such as phagocytes and other cell types containing cytotoxic activity due to the presence of peptides and phenoloxydase [20]. Non-self recognition is also an important aspect of the immune biology of these animals, relying mostly on lectins and major histocompatibility complex molecules. More recently, the presence of a hematopoietic system homologous to those of vertebrates have also been described, where a myeloid cell lineage is generated in the endostyle. Additionally, ascidians produce myeloid phagocytes and other lineages typical of invertebrates, such as amebocytes and large phagocytes [21]. In the last decade, many other species were added to the list of biological models, and the range and depth of knowledge regarding the mechanisms controlling development and the immune response has grown exponentially.

The immune system of ascidians is also involved in their associations with bacteria. In the gut, variable region-containing chitin-binding proteins (VCBP) are relevant to recognize and maintain the microbiome [22]. Moreover, as in mammals, the mucus lining the intestine is rich in chitin fibrils that bind to VCBPs and are produced during embryogenesis even before they start feeding. VCPBs, in turn, influence biofilm formation associated with the mucus, thus modulating the development and composition of the microbiota by favoring certain lineages, such as *Pseudoalteromonas* sp. and *Shewanella* sp. [23]. Yet, the interplay of the microbiome and immune system, as well as their potential adaptive role for the ascidian, especially as producers of toxic secondary metabolites, is not well understood. Furthermore, a wide range of these compounds act as anti-viral agents or as repellents against foulants, predators, and competitors [4], conferring the animal additional immune protection, because some of the compounds are produced by tunicate hemolymph cells [24].

Ascidians as marine holobionts
The holobiont concept has recently emerged as a new field in biology, in which macroorganisms and their associated-microbial community are considered complex ecosystems. Accordingly, the host and its associated-microbiota establish relationships, contributing to each other with defense, nutrition, protection, immunity and development of the host. The microbial community directly influences the function and even health of such ecosystem unit, therefore, they should be considered together for investigations regarding biological, ecological and evolutionary aspects [25].

In the marine environment, holobionts are further integrated into larger communities, such as coral reefs or kelp

forests. The water increases microbial connectivity and chemical signaling between organisms in any aquatic environment. Particularly in the case of filter-feeding animals, they are continuously exposed and surrounded by seawater and microbes. As holobionts, ascidians can provide an attractive environment for the development of diverse and abundant microbiota. Its tunic is composed mainly of polysaccharides – there is a carbohydrate polymer called tunicin, an animal cellulose – and proteins – such as collagen, elastin, pseudoelastin and/or hydroxypyroline [26]. The ecological niche promoted by tunicates can be a rich source of nutrients for the development of microorganisms.

There are few studies describing the intimate relationships between ascidians and their microbiota. One example is the ammonia-oxidizing archaea, that perform nitrogen recycling processes through nitrification. This recovery happens through ammonia resulting from nitrogenous ascidian processes. Ascidians provide nitrogen input and promote an oxygen-rich environment protected from high radiation in shallow reefs [27], maintaining the nitrogen cycle. In return, microorganisms may offer their host with growth factors, enzymes for digestion, secondary metabolites for the defense and nutrition, besides supporting them in colonizing new environments, especially those with pollutants or heavy metals [28,29]. Another example is related to metal accumulation in ascidians. Although not well understood, there are some evidences of the involvement of bacteria in such process. Two bacterial genera, *Pseudomonas* and *Ralstonia*, were found in high abundance into vanadium-rich ascidians, particularly in the pharynx, which is a tissue involved in vanadium absorption [30]. In addition, cyclic peptides produced by bacteria isolated from ascidians have shown metal chelating properties, suggesting their involvement in metal transporting or accumulation.

Symbiosis between didemnid-tunicates and the cyanobacteria *Prochloron* spp. is the most well-documented interaction among these organisms. The microorganism, which may establish different degrees of association with the invertebrate – from a simple biofilm in the tunic to intracellular symbiosis – provides the host with fixed C and recycles N, and is frequently essential for its survival. Besides its nutritional function, this symbiosis seems to be also driven by the secondary metabolism of the cyanobacterium, as shown by production of antioxidant mycosporine-like amino acids by *Prochloron didemni* in favor of *Lissoclinum patella*, allowing its host survival under high UV conditions [31]. However, even in this case, phylogenetic analyses indicate no co-evolution or co-speciation between symbiont and host [32,33]. In contrast, the other symbiotic relationship kept by *L. patella* with the Alphaproteobacteria *Candidatus* Endolissoclinum faulkneri, the likely producer of cytotoxic macrolides patellazoles, appears to be based on different grounds. These uncultivable bacteria show excessive genome reduction, *i.e.*,

loss of non-essential genes and a sizable cut of the genome occupied by the patellazole gene cluster. These are convincing signs of long-term maintenance of the relationship and, moreover, that secondary metabolism of the microbe is somehow indispensable for the symbiosis [34,35]. In fact, the didemnid-*Prochloron* symbiosis was also studied as a model to assess how this interaction may drive the metabolic diversity. In this case, cyanobactin gene clusters were compared among various *Prochloron* lineages from different didemnid hosts, showing that core peptides and enzymes may be swapped in the biosynthetic pathway, leading to functional modifications in the final product. This mechanism enables an accelerated alteration of secondary metabolism, when compared to a typical evolutionary process of a specialized biochemical pathway [36].

The ascidian microbiome

In the past, the ascidian microbiome was studied using classical approaches, isolating associated microorganisms in laboratory conditions. However, it has been generally accepted that, by using culture-dependent techniques, only a small fraction of microorganisms can be assessed. Bacteria, archaea and fungi have been isolated from ascidians. Among these taxa, bacterial phyla are the most abundant, with a high degree of diversity. In fact, six bacterial phyla have already been reported, including Actinobacteria (18 genera from 12 families), Proteobacteria (16 genera from 11 families), Firmicutes (6 genera from 4 families), Cyanobacteria (2 genera from 2 families), Bacteroidetes (2 genera from 2 families) and Verrucomicrobia (1 genus). Three fungal phyla were also described, including Ascomycota (24 genera from 17 families), Zygomycota (2 genera from 2 families) and Basidiomycota (1 genus).

The development of advanced molecular biology techniques, based on metagenomics and community sequencing libraries, has allowed a more representative overview of the microbiota of holobiont systems [37]. In general, Proteobacteria is by far the most abundant bacterial phyla described in ascidians, representing more than half of the total microbiota in most ascidians assessed by methods such as next-generation sequencing. This phylum is diversified among ascidians, where Alpha- and Gammaproteobacteria are the classes most frequently detected. Beta- and Deltaproteobacteria have also been described in some ascidians, at lower abundance. Several additional bacterial phyla have been found in different ascidian species, including Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes and Spirochaetes [38,39].

A species-specific core-microbiome, not yet well defined, can be found in a particular ascidian species during all of its life stages, as well as across different individuals, even from different geographical regions. Samples of ascidians from the Aplousobranchia, Phlebobranchia, and Stolidobranchia orders obtained from California, Florida, Papua New Guinea, Vanuatu, and Fiji were compared for their microbiome, and

the resulting cluster analyses showed significant grouping based on host species, in which variation of the group was not correlated to the geographical location, but, in fact, to the species themselves [38]. Another study with the ascidian *Didemnum fulgens* showed the presence of stable and unique bacterial communities composed mainly by Alpha and Gammaproteobacteria, as well as by a few Cyanobacteria and Acidobacteria, at least, partially inherited by their progeny by vertical transmission despite temperature variation [40]. A core community for *Styela plicata* and *Herdmania momus* was also observed, including microorganisms with capabilities of ammonia oxidization, carbon fixing and metal chelating [28]. In this case, the core community accounts only for approximately 0.1% of bacterial OTUs, but with an expressive abundance of 26–36.2% of the sequence reads. In contrast, a dynamic and abundant community (>20% of OTUs reads) related to location and season was also observed [28].

Source of compounds with pharmacological interest

Tunicates have long been known to yield remarkable compounds with noteworthy pharmacological potential. To date, more than 1000 chemical structures isolated from these organisms have been described [41]. Among these, over 70% of them are alkaloids, including indole alkaloids as the most abundant (around 50%), followed by pyrocridine (~20%), beta-carboline (~8%) and indolocarbazole (staurosporines) (~5%). Overall, several alkaloids have shown anticancer properties, which makes ascidians as promising sources of such compounds. In fact, more than 60% of compounds isolated from ascidians reported between 1994 and 2014 have been described for their anticancer properties [7].

A very good example of the pharmacological potential of alkaloids from tunicates are the cytotoxic tetrahydroisoquinoline alkaloids named ecteinascidins (ETs), isolated from *Ecteinascidia turbinata* in 1969 [42], which marks the beginning of chemical investigation of these organisms. Interestingly, the complete structure elucidation of such molecules was only achieved about 20 years later [11], due to their high complexity. Among the most active substances, ecteinascidin 743 or ET-743, better known now as trabectedin, was revealed to be cytotoxic against a great variety of cell lines and xenograft models, with motivating results obtained for sarcoma models. Trabedtedin was developed by PharmaMar and approved for the treatment of advanced soft tissue sarcoma in 2007 and, later, for relapsed ovarian cancer [43]. In this context, it is only fair to credit this unique natural molecule, trabectedin, for paving the way and confirming the pharmacological potential of ascidians, as it was the first marine natural product to be approved for clinical use as anticancer drug.

Another interesting class of molecules is peptides, which actually comprise only 4% of the chemical classes described from tunicates. Nevertheless, they have been proved to be pharmacologically very relevant. This is the case of the didemnins, cyclic depsipeptides isolated from extracts of *Trididemnum solidum*. Didemnim B, particularly, showed relevant anticancer, immunomodulatory and antimicrobial activities [44]. This molecule entered clinical trials in 1986 as the first truly marine natural compound to achieve such a status, however elevated toxicity and lack of efficacy had it withdrawn from trials by mid-1990s [45]. Nevertheless, an analog of didemnin B, known as aplidin or plitidepsin, obtained from *Aplidium albicans* emerged with encouraging results, since it showed higher potency and lacked cardiotoxicity. Aplidin entered clinical trials in 1999, directly benefiting from preclinical and clinical data generated in studies with didemnin B and has been approved, in 2018, for the treatment of refractory multiple myeloma when associated with dexamethasone, in Australia [46,47].

Tunicates are also a rich source of compounds with anti-infectious properties, as nearly 21% of molecules have been recognized for their antibacterial, antiviral and antiprotozoal activities. Interestingly, butenolides, antibacterial lactones isolated from *Pseudodistoma antinboja* showed a selective activity against Gram-positive bacteria, actually giving better response in comparison to the currently approved drugs vancomycin and linezolid, being effective also against drug-resistant strains [48]. Moreover, albopunctatone, an anthrone-anthraquinone isolated from *Didemnum albopunctatum was described with antimalarial activity against chloroquine-resistant strains of Plasmodium falciparum [49]. However, despite promising results, no anti-infectious substance derived from tunicates has reached clinical trials.*

Despite the unquestionable pharmacological potential of compounds isolated from tunicates, there are strong evidences that nearly 8% of secondary metabolites originally described from ascidians are, in fact, produced by associated microorganisms [48]. However, indirect evidences hint their involvement in many more [50]. Numerous metabolites that have been initially discovered from ascidians are similar or even alike compounds later isolated from bacteria [6]. Table 1 presents a list of compounds firstly sourced from ascidians that are indeed produced by their associated bacteria. Furthermore, the table also includes a description of the pharmacological properties attributed to each metabolite and an explanation of their biological function for the host. Trabectedin, for instance, firstly sourced from *E. turbinata* [51], has been recently demonstrated by meta-omic approaches that it is actually produced by the obligatory symbiotic, yet uncultivable, Gammaproteobacteria *Candidatus* Endoecteinascia frumentensis [52]. Another example can be illustrated by didemnin B originally isolated from *T. solidum* [44], which was then found in extracts obtained from the free-living Alphaproteobacteria *Tristella mobilis* and *T. bauzanensis* [53]. Although these bacteria have not been necessarily

Table 1. Secondary metabolites produced by associated-microorganisms originally described from ascidians, their pharmacological properties and putative biological function.

Metabolite	Ascidian	Microorganism	Pharmacological property	Putative biological function of metabolite	Ref.
Didemnin B (Cyclic peptides)	*Trididemnum solidum*	*Tristella mobilis* and *T. bauzanensis*	Anti-cancer properties, however high toxicity.	Deterrent of predators. Tests with fish predators showed regurgitation when fed with mosquito larvae (mimicking ascidian larvae) containing synthetic analogs of Didemnin B.	[55]
Ascidiacyclamide (cyanobactin)	*Lissoclinum patella*	*Prochloron* spp.	Cytotoxicity against tumor cell lines	It was isolated and characterized as a biscopper(II) complex of ascidiacyclamide. This complex has particular affinity for carbonate, which suggests that it could be involved in the activation and transport of CO_2 in specific biochemical processes. However, cyanobactins, in general, present metal-chelating properties, which suggest that they could be involved in metals transport and storage, or detoxification, once concentration of metals in ascidians are usually much higher than in the sea water.	[56]
Rossinone and Meridianins	*Aplidium fuegiense, A. falklandicum A. meridianum* and *Synoicum adareanum*	Possibly of bacterial origin	Cytotoxicity and kinase inhibitory activity	Chemical defense (deterrent) against predators (sea star *Odontaster validus* and amphipod *Cheirimedon femoratus*). Some meridianins also showed a potential activity against a sympatric bacterial fouling.	[57]
Patellazoles	*Lissoclinum patella*	*Candidatus Endolissoclinum faulkneri*	Cytotoxicity against HCT-116 tumor cells	The genome of this proteobacteria is extremely reduced, and therefore it cannot live independently of the ascidian. However, the genes involved in production of patellazoles were maintained, which suggests a strong relationship between the organisms. The strong cytotoxicity indicates that these compounds may have a protective role.	[58]
Divamides	*Didmnum molle*	*Prochloron didemni*	Anti-HIV	The biological role of divamides in ascidians is still unknown. The anti-viral property and the antibiotic activity of analogs (lanthipeptides) suggests that divamides can be involved in protection against pathogens, such as bacteria and virus.	[59]

linked to the ascidian, the biosynthetic gene cluster of didemnins has been elucidated within the genome of *T. mobilis* [54], offering compelling indication that a microbial association would be in play, when the didemnins were firstly obtained among the invertebrate tissues.

Concluding remarks

Ascidians have long been considered model organisms for studies that range from developmental biology to immune system evolution and function. As filter-feeders, with a large perforated pharynx, these organisms are suitable hosts for a diverse associated biota, including both macro and microorganisms. This potentially rich microbiome is directly related with a diversified chemical profile. In fact, ascidians have been regarded as rich sources of compounds with varied pharmacological properties. More recently, many studies revealed that most of the interesting secondary metabolites are, in fact, produced by associated microorganisms, especially bacteria. Hence, the ecological and biological aspects of ascidians seem to have favored associations with microorganism capable of producing toxic compounds.

With the emergence of the holobiont paradigm, these animals also became interesting models to assess the interplay between host and its microbiome components, in order to understand how this chemical diversity evolved in an adaptive context. Recent improvements on techniques such as metagenomics, as well as metabolomics, microscopy and single-cell approaches, have taken an imperative role in the investigation of holobionts. Nevertheless, despite the continuous progress and increasing number of researchers dedicated at assessing a wide range of questions concerning the relationship between microbes, hosts and metabolome, there are still lots of crucial blanks to fill. Therefore, interdisciplinary approaches directed towards the investigations of holobiont models are strongly encouraged to address comprehensively knowledge to many different fields.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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