Allosteric Modulation of Ligand Gated Ion Channels by Ivermectin

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Received December 2, 2013
Accepted December 11, 2013

Summary
Ivermectin acts as a positive allosteric regulator of several ligand-gated channels including the glutamate-gated chloride channel (GluCls), γ-aminobutyric acid type-A receptor, glycine receptor, neuronal α7-nicotinic receptor and purinergic P2X4 receptor. In most of the ivermectin-sensitive channels, the effects of ivermectin include the potentiation of agonist-induced currents at low concentrations and channel opening at higher concentrations. Based on mutagenesis, electrophysiological recordings and functional analysis of chimeras between ivermectin-sensitive and ivermectin-insensitive receptors, it has been concluded that ivermectin acts by insertion between transmembrane helices. The three-dimensional structure of C. elegans GluCl complexed with ivermectin has revealed the details of the ivermectin-binding site, however, no generic motif of amino acids could accurately predict ivermectin binding site for other ligand gated channels. Here, we will review what is currently known about ivermectin binding and modulation of Cys-loop receptor family of ligand-gated ion channels and what are the critical structural determinants underlying potentiation of the P2X4 receptor channel.

Key words
Cys-loop receptor family • Purinergic P2X4 receptor • Ivermectin • Transmembrane domain

Introduction
Ivermectin is a high molecular weight compound that is highly lipophilic, exhibiting very low water solubility of 4 mg/l (Bassissi et al. 2004). It is synthesized by the selective hydrogenation of avermectin B1, a macrolcyclic lactone produced by the soil bacterium Streptomyces avermitilis. Ivermectin comprises a mixture of 22, 23-dihydroavermectin B1a and B1b in a 80:20 ratio. These compounds differ in structure only at C25 (Fig. 1), a part of the molecule not involved in ivermectin binding (Hibbs and Gouaux 2011). Ivermectin, has been used with success in veterinary medicine since 1981 and more recently in human (Taylor et al. 1990). It has become a widely used anti-helminthic and insecticidal drug and is the drug of choice for the treatment and prevention of human onchocerciasis, commonly known as river blindness, and other human filarial infections (Cupp et al. 2011). Ivermectin kills the nematode Caenorhabditis elegans at therapeutic concentrations by activating glutamate-gated chloride channels (GluCls) that contain α-type channel subunits (Cully et al. 1994, Dent et al. 1997). Positive allosteric modulators of muscle or neuronal GluCl receptors have the potential to disrupt the rhythmic transmission required for pharyngeal pumping, causing pharynx paralysis and eventually death of worms due to starvation (Dent et al. 1997, Keane and Avery 2003). Some nematodes are resistant to ivermectin and analysis of their GluCl receptors helped to identify structural determinants of ivermectin-sensitivity (Dent et al. 2000). Specific ivermectin binding sites have also been identified in the mammalian brain (Huang and Casida 1997); however, the affinity of ivermectin for

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these sites is lower by a factor of about 100 as compared to nematode or insect membranes, which may account for its remarkable success in eliminating parasitic nematodes and insects from mammals.

Fig. 1. Formula of ivermectin (IVM) molecule. IVM is a large molecule of approximately 870 kDa. It possesses a 16-membered macrocyclic lactone ring with a disaccharide substituent at C-13, this sugar moiety together with the hydroxy-group at C-5, may be the significant structural determinants of antihelmintic and insecticidal activity. The first three-dimensional structure of an eukaryotic ligand-gated ion channel, C. elegans GLC-1, complexed with ivermectin (Hibbs and Gouaux 2011) has revealed that the benzofuran head of ivermectin is orientated toward the channel pore and the spiroketal is oriented toward the cytoplasm. The disaccharide remains outside the binding cleft in contact with the surrounding lipids and the extracellular protein loop.

Ivermectin also acts as a positive allosteric regulator of several ligand-gated ion channels in vertebrates. Submicromolar concentrations of ivermectin activate or modulate the γ-aminobutyric acid type-A receptor (GABAAR) (Krusek and Zemková 1994), glycine receptor (GlyR) (Shan et al. 2001) and neuronal α7-nicotinic receptor (nAChR) (Krause et al. 1998). The Cys-loop receptors are formed by the assembly of five of the same (homomer) or different (heteromer) subunits. Each subunit consists of a large extracellular N-terminal domain, four membrane-spanning helices (M1–M4) that constitute the transmembrane domain and a short extracellular C-terminal domain. The conventional orthosteric agonist binding site is located within the extracellular domain at the interface between two adjacent subunits. Ivermectin also potentiates purinergic P2X receptors (P2X1-7) are ATP-gated cation channels permeable to Na+, K+, Ca2+, and small organic cations. These channels are comprised of three subunits, and single P2XR subunit is composed of two transmembrane domains (TM), a large extracellular ligand-binding loop, and intracellularly located N- and C-termini (Ralevic and Burnstock 1998).

Other effects of ivermectin include the increase of Ca2+ release from sarcoplasmic reticulum via activation of ryanodine receptors and inhibition of Ca2+ uptake by sarcoplasmic reticulum Ca2+ pumps (Ahern et al. 1999), and activation of histamine-gated chloride channels in Drosophila, channels closely related to GlyR α-subunits (Zheng et al. 2002).

In most of ivermectin-sensitive Cys-loop channels, the effects of ivermectin include the potentiation of agonist-induced currents at low concentrations, channel opening at higher concentrations, and much slower direct activation of channels as compared to classical transmitters. Once open, the channels remain in open state for a very long time, indicating that ivermectin and agonists activate the channels via structurally distinct mechanisms (Shan et al. 2001, Lynagh and Lynch 2010, Lynagh et al. 2011). It has been suggested that maximal activation of the Cys-loop channels may require as few as two bound ivermectin molecules and that ivermectin maximally activates heteromeric GlyRs by binding to two interfaces between different subunits (Lynagh and Lynch 2010).

Single channel kinetic analysis revealed that application of submicromolar concentrations of 22,23-dihydroavermectin B1a on outside-out patches from collagenase-treated muscle membranes of Ascaris suum initially induced the activation of a single channel, although the number of active channels increased over time to the extent that single channel activations could no longer be detected (Martin and Pennington 1989). The delay between avermectin application and channel opening is long (greater than 15 s), suggesting that due to its lipophilic nature ivermectin accumulates in the membrane and binds reversibly (i.e. weakly) to its site (Martin and Pennington 1989). The P2X4R channel cannot be directly opened with ivermectin, but is also modulated reversibly and ivermectin has two concentration dependent effects on P2X4R: it potentiates ATP-induced response at low, and increases receptor sensitivity to agonists at higher concentrations (Khakh et al. 1999, Priel and Silberberg 2004).

Many functional studies based on analysis of

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ivermectin-resistant channels in nematodes (Dent et al. 2000, Njue et al. 2004, Dufour et al. 2013), and chimeras between ivermectin-sensitive and ivermectin-insensitive vertebrate receptors (Jelinkova et al. 2006, Silberberg et al. 2007) predicted that ivermectin acts by insertion between transmembrane helices of ligand-gated channels. The crystal structure of C. elegans GluCl in open state complexed with ivermectin has provided a clear evidence that ivermectin interacts with an allosteric site in the transmembrane domain (Hibbs and Gouaux 2011). Unique features of the three-dimensional structure of a GluCl with bound ivermectin contributed largely to the knowledge about ivermectin sensitivity of the entire Cys-loop ligand-gated ion channel superfamily recently reviewed by Wolstenholme (2012). The structure of zebrafish P2X4R (zP2X4R) in open state with bound ATP has been already solved (Hattori and Gouaux 2012), however, the ivermectin binding site at P2X4R is still to be identified.

Glutamate-gated Cl- channels

GluCl channels are found exclusively in invertebrates and belong to the pentameric Cys-loop receptor family of ligand-gated ion channels. GluCl channels are either directly activated or their responses to glutamate are potentiated by ivermectin (Cully et al. 1994, 1996). Analysis of ivermectin-resistant channels (Dent et al. 2000) has shown that sequence variation in the α-subunit of a GluCl confers avermectin (AVR) resistance of nematodes. In C. elegans, α-type subunits are encoded by a family of genes including glc-1 (encoding GLC-1/GluClα) and avr-15 (encoding AVR-15/GluClα2). Severe loss-of-function mutations in glc-1 or avr-15 did not make worms resistant to ivermectin, however, simultaneous mutation of three genes encoding GluCl α-type subunits conferred high-level resistance to ivermectin. These results suggested that the ability of ivermectin to target several members of a multigene family may decrease the rate at which resistance evolves (Dent et al. 2000).

The first three-dimensional structure of an eukaryotic ligand-gated ion channel, C. elegans GLC-1, complexed with ivermectin (Hibbs and Gouaux 2011) has revealed the detailed structure of both the glutamate- and ivermectin-binding sites. Crystal shows that the GluCl receptor (GluClR) subunits are arranged with five-fold symmetry, such that the M2 helices from each subunit are located centrally, forming the channel pore. The amino acid composition of the M2 helix thus determines the ion selectivity and conductance properties of the channel. Ivermectin binds to the GluCl receptor in the outer half of the membrane. The ivermectin-binding site is located in a cleft between M3 and M1 helices of two adjacent subunits. Ivermectin also makes contact with M2, which lines the pore of ion channel, through its disaccharide moiety and the M2-M3 loop. Crystal also identified three hydrogen bonds linking ivermectin and the transmembrane residues L218 (M1), S260 (M2), and T285 (M3). The benzofuran head of ivermectin is orientated toward the pore, the spiroketal is oriented toward the cytoplasm and the disaccharide remains outside the binding cleft in contact with the surrounding lipids and protein surface. There are twelve additional van der Waals interactions between ivermectin backbone macrocycle and M3, M2, and M1 residues. Ivermectin tends to push the membrane-spanning regions of the subunits apart and stabilizes an open-pore conformation. The contacts between ivermectin with the M2-M3 loop and other parts of the extracellular domain may transmit the allosteric signal to the ligand-binding site. Thus, ivermectin binding to this site induces a global conformational change that propagates from the transmembrane domain to the neurotransmitter binding site, suggesting a mechanism by which ivermectin potentiates neurotransmitter-gated currents (Hibbs and Gouaux 2011).

There have been attempts to use GluCls and develop them as tools for the specific silencing of defined vertebrate neurons by adding low concentrations of ivermectin (Slimko et al. 2002). Expression of the C. elegans GLC-1 and GLC-2 subunits in mammalian neurons using recombinant virus vectors results in the formation of functional GluCls, which can then be selectively activated by injecting ivermectin (Lerchner et al. 2007). The subunits have been modified to encode GFP as a marker and engineered to eliminate activation by glutamate (Slimko et al. 2002, Lin et al. 2011). Such methods have been used to reduce the level of interneural aggression by single intraperitoneal injection of 10 mg/kg of ivermectin (Lerchner et al. 2007).

The structure of ivermectin binding site discovered for C. elegans GLC-1 channel confirmed previous predictions of site-directed mutagenesis experiments (Njue et al. 2004, Yamaguchi et al. 2012). Mutagenesis experiments also indicated an overlap between the ivermectin-binding site and that of volatile anesthetics and other drugs that act at mammalian
GABAA and glycine receptors (Lynagh and Lynch 2010, Lynagh et al. 2011). This structure is therefore of great importance to pharmacology of mammalian receptors because lipophilic modulators of other Cys-loop receptors may exploit a similar mechanism of interaction, including the neurosteroids at the GABAA (Miller and Smart 2010) and glutamate receptors (Korinek et al. 2011), and cholesterol at the muscle nAChR (Barrantes 2004).

**Glycine receptor**

In vertebrates, ivermectin also activates GlyRs although less potently than GluClRs (Adelsberger et al. 2000, Shan et al. 2001). It is clear that ivermectin and glycine activate the GlyR via structurally distinct mechanisms because ivermectin potently activates GlyRs that are completely desensitized to glycine and mutations that eliminate glycine sensitivity frequently have little or no effect on ivermectin sensitivity and vice versa (Shan et al. 2001, Lynagh and Lynch 2010, Lynagh et al. 2011). Ivermectin- and glycine-mediated currents also exhibit distinct pharmacological properties (Shan et al. 2001).

The understanding of ivermectin binding to GlyRs and activation mechanisms is advanced due the publication of a crystal structure of ivermectin bound to the C. elegans a GluClR (Hibbs and Gouaux 2011) and a systematic site-directed mutagenesis study on ivermectin sensitivity determinants at the a1 GlyR (Lynagh et al. 2011). Mutagenesis experiments, combined with a homology model based on the C. elegans a GluClR, revealed a similar binding orientation for ivermectin between M1 and M3 helices in GlyRs. In the a1 GlyR, A288F (M3) and P230W (M1) mutations are possible determinants of ivermectin binding as they decreased both the direct agonistic and (M1) mutations are possible determinants of ivermectin binding conformation almost identical to that at the unmutated a1 GlyR (Lynagh et al. 2011). However, site-directed mutagenesis studies and amino acid sequence analysis on other ivermectin-sensitive anionic Cys-loop receptors revealed that these two residues are equivalent to two of the a GluClR residues identified by Hibbs and Gouaux as being involved in the van der Waals interactions with the ivermectin backbone macrocycle, which implies that hydrogen bonds with residues equivalent to S260 and T285 are not required for high ivermectin sensitivity at either the a1 GlyR or three other high ivermectin-affinity GluClRs (Lynagh and Lynch 2012). As the hydrogen bonds between the hydroxyl groups of ivermectin with the L218 or equivalent residues is via the protein backbone carbonyl, its existence cannot be readily tested by site-directed mutagenesis. Thus, site-directed mutagenesis and voltage-clamp electrophysiology that have been employed to probe the binding site for ivermectin in a1 GlyRs indicate that hydrogen bonds between ivermectin and channel might not be essential for high ivermectin potency. It has been suggested that it may be space at the M1-M3 interface, rather than hydrogen bonds, that most crucially determine ivermectin potency at Cys-loop receptors (Lynagh and Lynch 2012).

**GABAA receptor**

The GABAAR is characterized by multiple binding sites which modulate GABA responses (Sieghart 1995), the most important being the binding site for pharmacologically and clinically important drugs such as benzodiazepines, barbiturates, bicuculline and picrotoxin. The application of ivermectin in nanomolar concentrations to the native GABAAR in embryonic mouse hippocampal neurons potentiated the GABA-induced Cl⁻ currents (Fig. 2) and reduced the GABA EC₅₀ value. Modulation of the GABA responses by ivermectin did not interfere with the potentiation induced by diazepam and pentobarbital or with the sensitivity to blockade by bicuculline, picrotoxin and Zn²⁺, indicating that ivermectin binds to a specific site on the GABAAR receptor and allosterically enhances the affinity of the GABA binding site (Krusek and Zemkova 1994). The final effect of ivermectin was irreversible, at least within the time frame of several minutes required for electrophysiological experiments. However, covalent interactions between ivermectin and its site are not evident in the GluClR crystal structure (Hibbs and Gouaux 2011), nor would they be expected to exist given the chemical properties of ivermectin. The alternative explanation therefore is that ivermectin partitions in to the membrane, due to its lipophilic nature, where it reaches a high local concentration and thus much of the binding energy of ivermectin could derive from the non-specific free energy of membrane partitioning, with the actual ligand-channel interaction being quite weak and thereby reversible (Lee and MacKinnon 2004).

In voltage-clamped Xenopus oocytes into which
isolated mRNA from the chick brain had been injected, submicromolar concentrations of avermectin B1a allosterically potentiated GABA-induced currents (Sigel and Baur 1987). Avermectin B1a also potentiates the GABA-gated chloride channel of cultured cerebellar granule neurons (Huang and Casida 1997) and GABAergic transmission in invertebrates (Duce and Scott 1985). At concentrations above 10 μM, avermectin B1a directly activates the Cl− channel in GABA-sensitive dorsal root ganglion neurones of rats and cats (Robertson 1989). These currents are sensitive to blockade by bicuculline and picrotoxin, indicating that they are due to activation of GABAARs expressed in human embryonic kidney (HEK) 293 cells. Whole-cell recording showed that maximal activation of the channel occurred with GABA concentrations of 10 μM or 20 μM avermectin and that both stimulated about the same current amplitudes but the rise-time and decay of avermectin-activated currents were about 500 times slower than those of GABA-activated currents. In contrast to activation with GABA, no desensitization of the receptor was observed with avermectin. The single channel kinetic analysis revealed that channel openings were markedly longer in the presence of avermectin, indicating that GABA and avermectin activated the channel independently resulting in different kinetic properties (Adelsberger et al. 2000).

The mechanism of potentiation of GABAARs by ivermectin has been suggested to be similar as for potentiation of other Cys-loop receptor family of ligand-gated ion channels (Lynagh and Lynch 2012), involving separation of M3 and M1 and coupling of ivermectin and M2 or M2 and M3, which might be required for direct activation of channel. Similar mechanisms might also underlie the potentiation and activation of GABAARs and GlyRs by the anaesthetic etomidate (Pistis et al. 1997), which is supposed to bind at subunit interfaces in the same cleft as occupied by ivermectin (Li et al. 2006, Chiara et al. 2012).

**Nicotinic acetylcholine receptor**

The excitatory ACh-gated cationic channels, nicotinic acetylcholine receptors (nAChRs), are also members of the Cys-loop family of ligand-gated ion channels. They are important neurotransmitter receptor subtypes in both vertebrate and invertebrate species (Millar and Denholm 2007). The nAChR has close structural similarity to GluClR and is also the target site for ivermectin. Homomeric α7 nAChR are potentiated by low concentrations of ivermectin (Krause et al. 1998), but ivermectin has been also shown to potentiate α7 nAChRs activity at high (30 μM) concentrations (Collins and Millar 2010).

By testing the influence of ivermectin on chimeras containing domains from the nAChR α7 subunit and the ivermectin-insensitive 5-hydroxytryptamine (5-HT) type 3 (5-HT(3)) receptors, it has been shown that the transmembrane helices play a critical role in allosteric modulation by ivermectin (Collins and Millar 2010). Single-point mutation studies of transmembrane domains of the α7 nAChRs subunit showed that four mutations (A225D, Q272V, T456Y and C459Y) caused a significant reduction in the potentiation effect of ivermectin when compared with WT and three mutations (S222M, M253L, and S276V located in TM1, TM2, and TM3, respectively) converted ivermectin from a positive allosteric modulator into an antagonist. Computer docking simulations provide support for the hypothesis that these seven mutations that influence allosteric modulation by ivermectin are located near a predicted intrasubunit transmembrane cavity which could bind ivermectin (Collins and Millar 2010).
**P2X4 receptor**

The P2X4 receptor, but not other subtype of ionotropic ATP-gated ion channels, is sensitive to ivermectin (Khakh et al. 1999). The extracellularly applied ivermectin potentiates ATP-induced responses but it does not produce direct activation of the channel. The effect of ivermectin is concentration- and time-dependent; at low concentrations ivermectin increases the maximal current amplitude, and at higher concentrations increases the sensitivity to ATP and the partial agonist αβ-meATP, and prolongs deactivation of the receptor after the removal of agonist (Khakh et al. 1999, Priel and Silberberg 2004, Jelinkova et al. 2006). Consequently it has been postulated that the two distinct effects of ivermectin are due to binding at two distinct sites. The suggested hypothesis is that when ivermectin binds the higher affinity binding site, i.e. increases response by increasing the probability of channel opening, reducing the probability of channel desensitization, and when it binds a lower affinity binding site, it increases deactivation time by stabilizing the open conformation of the receptor (Priel and Silberberg 2004). Experiments with chimeric receptors containing domains from ivermectin-sensitive P2X4R and the ivermectin-insensitive P2X2 subunit provided evidence that the transmembrane domains play a critical role in allosteric modulation by ivermectin (Jelinkova et al. 2006). Using alanine and cysteine scanning mutagenesis, we attempted to identify transmembrane residues responsible for specific ivermectin binding. We found that the following P2X4 receptor residues could participate in the recognition of ivermectin molecule: R33, Q36, L40, V43, V47 and W50 residues of TM1, and N338, G342, L346, A349, C353, and I356 residues of TM2 (Jelinkova et al. 2008). These predominantly non-polar residues could be lipid-oriented and are also present in the ivermectin-sensitive *Schistosoma mansoni* P2X subunit (*schP2XR*) (Agboh et al. 2004). However, the *schP2XR* does not undergo as large potentiation due to ivermectin as does the P2X4R, and also lacks major features of the ivermectin effect on the P2X4R; ivermectin had little effect on the EC50 of the agonist and no effect on the observed deactivation rates (Agboh et al. 2004). Thus, *schP2XR* may serve a role in future studies regarding the action of ivermectin on P2X4Rs and may be useful in identifying the existence of binding sites specific to each of the observed effects of ivermectin.

The location of the ivermectin binding site has not yet been addressed in the context of the recent crystal structures of a zfP2X4R in an apo, closed channel state (Kawate et al. 2009) and open state with bound ATP (Hattori and Gouaux 2012). Provided that ivermectin binding site in P2X4R occupies about 2 turns of helix on the upper part of transmembrane domains (Fig. 3), the residues V43, V47 and W50 in TM1 and N338, G342 and L346 in TM2 are the most serious candidates for mediating ivermectin binding. The remaining mutations in the lower part of TM1 and TM2 helices apparently did not disrupt ivermectin affinity, but rather the efficacy with which ivermectin potentiates the receptor. These nonpolar residues are localized on the same side of their helices. Furthermore, the three-dimensional models of the ivermectin molecule and TM1 fragment of P2X4R (Fig. 3) indicate that these residues might be accessible by large ivermectin molecule simultaneously. In accordance with this, none of the single mutants in this region fully abolished ivermectin effects on current amplitude and the rate of deactivation (Silberberg et al. 2007, Jelinkova et al. 2008). These results are consistent with a hypothesis that ivermectin inserts between the two neighboring subunits of P2X4R channel in the membrane and interferes with the molecular rearrangement in the TM domains involved in channel gating, similarly as found for the Cys-loop receptor family of ligand-gated ion channels (Hibbs and Gouaux 2011). Accordingly, there should be three potential binding sites for ivermectin in the P2X4R because there are three clefts.
between subunits. In that scenario, occupancy of the first binding site is sufficient to show effects on current amplitudes, whereas occupancy of additional binding site(s) is required for the effects on the rate of deactivation. We may also speculate that the insertion of one ivermectin molecule decreases the affinity of the residual binding sites for ivermectin. Finally, it appears that the position of nonpolar residues rather than their receptor specificity accounts for the potentiating effects of ivermectin, and that the P2X4R-specific W50 residue is required for the full development of ivermectin effect on current deactivation (Jelinkova et al. 2006). Ivermectin-induced changes in agonist binding site and receptor sensitivity (Khakh et al. 1999, Priel and Silberberg 2004, Jelinkova et al. 2006, Silberberg et al. 2007) might be due to changes in the conformation of the entire ectodomain of P2X4R as suggested for the Cys-loop receptor family (Lynagh and Lynch 2012).

Despite the apparent non-specific nature of ivermectin binding, ivermectin has been shown to selectively modulate the P2X4R and serves as an important tool to functionally distinguish the receptor from other P2XRs (Coddou et al. 2011, Vavra et al. 2011).

**Conclusion**

Several allosteric sites have been identified within the family of Cys-loop receptors (Sieghart 1995) and P2X receptors (Coddou et al. 2011) and with the contextual knowledge about ivermectin action and binding, a detailed picture of allosteric modulatory mechanisms of these important receptors is emerging. Positive allosteric modulators of ionotropic glutamate receptors are potential compounds for treatment of cognitive disorders, e.g. Alzheimer's disease (Norholm et al. 2013). Allosteric modulators of nAChRs have the potential to improve cognitive function and alleviate pain (Olsen et al. 2013), and positive allosteric modulators of P2X4 receptors have the potential to improve the learning and memory processes (Lorca et al. 2005). Thus, it is important to bring together the knowledge of how ivermectin modulates these channels, in order to facilitate the development of new ivermectin-based therapies in future.

**Conflict of Interest**

There is no conflict of interest.

**Acknowledgements**

This study was supported by the Internal Grant Agency of Academy of Sciences (Grant No.IAA500110910), the Grant Agency of the Czech Republic (P304/12/G069 and P304/12/P371), Grant Agency of Charles University (3446/2011), the project „BIOCEV – Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University“ (CZ.1.05/1.1.00/02.0109), from the European Regional Development Fund, and the Academy of Sciences of the Czech Republic (Research Project No. RVO 67985823).

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