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Inhibition of Bfl-1 with *N*-aryl maleimides

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ABSTRACT

High-throughput screening of 66,000 compounds using competitive binding of peptides comprising the BH3 domain to anti-apoptotic Bfl-1 led to the identification of 14 validated 'hits' as inhibitors of Bfl-1. *N*-Aryl maleimide **1** was among the validated 'hits'. A chemical library encompassing over 280 analogs of **1** was prepared following a two-step synthesis. Structure–activity studies for inhibition of Bfl-1 by analogs of *N*-aryl maleimide **1** revealed a preference for electron-withdrawing substituents in the *N*-aryl ring and hydrophilic amines appended to the maleimide core. Inhibitors of Bfl-1 are potential development candidates for anti-cancer therapeutics.

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The B-cell lymphoma/leukemia-2 (Bcl-2) family of proteins plays a prominent role in regulating programmed cell death (apoptosis). In humans, proteins within the Bcl-2 family having anti-apoptotic activity include Bcl-2, Bcl-X_L, Mcl-1, Bcl-W, Bcl-B, and Bfl-1.¹ Members of this group of proteins are commonly over-expressed in human cancers thereby promoting tumorigenesis.^{2,3} Additional groups of proteins within the Bcl-2 family have pro-apoptotic activity and include Bak, Bax, Bad, Bim, and Bid. Anti-apoptotic and pro-apoptotic proteins can dimerize, either activating, or antagonizing each other.⁴

Heterodimerization occurs at a hydrophobic pocket on the surface of anti-apoptotic proteins and a region of pro-apoptotic proteins called the BH3 region to neutralize the cytoprotective function of anti-apoptotic Bcl-2 family proteins. The BH3 region is comprised of an amphipathic α -helix of roughly 16–25 amino acids that binds to the hydrophobic region of the anti-apoptotic Bcl-2 family proteins.¹ Agents that mimic the BH3 region of pro-apoptotic proteins may be used to antagonize anti-apoptotic Bcl-2 proteins by binding to the hydrophobic pocket thus promoting cell death. Synthetic peptides mimicking the BH3 region have been shown to bind to the anti-apoptotic Bcl-2 proteins and initiate apoptosis.^{5,6}

Small molecule inhibitors of anti-apoptotic Bcl-2 members have been reported.⁷ Some natural product inhibitors include gossypol⁸ and epigallocatechin (EGCG).⁹ Some synthetic and semisynthetic inhibitors include ABT-737,¹⁰ GX15-070,¹¹ apogossypol,¹² and the commercially available thioxothiazolidine, BH3I-1.^{13,14} These agents act by binding to the hydrophobic pocket of anti-apoptotic

Bcl-2 proteins, mimicking BH3 peptides to block their cytoprotective effects. Some small molecules inhibit a broad range of the anti-apoptotic Bcl-2 proteins while others are more selective.

Bfl-1 is a NF- κ B-inducible member of the Bcl-2 family, that is highly expressed in immune cells.¹⁵ Defective apoptosis has been implicated in several autoimmune diseases, thus suggesting a therapeutic opportunity for agents that neutralize Bfl-1. In addition, Bfl-1 is highly expressed in some cancers accounting for resistance to selective Bcl-2/Bcl-X_L inhibitors that fail to neutralize this anti-apoptosis member of the Bcl-2 family. Based on these results, we decided to focus on small molecule inhibitors of Bfl-1 that interact at the BH3 binding site.

Herein, we describe the process used to identify and synthesize promising lead compounds for inhibition of the anti-apoptotic protein, Bfl-1. A high-throughput fluorescence polarization (FP) assay was developed to detect inhibition of Bfl-1 by small molecules.¹⁴ In this assay, inhibitors were incubated with glutathione-S-transferase (GST)-Bcl-2 anti-apoptotic fusion proteins.¹⁶ FP was measured after addition of an N-terminal fluorescein isothiocyanate (FITC) BH3 peptide (i.e., FITC-Ahx-EDIIRNIARHLAQVGDSMDR-CO₂H).^{5,17} Inhibitory potency was confirmed using a Lanthascreen time-resolved fluorescence resonance energy transfer (TR-FRET) assay that examined the same molecular interaction between the FITC-peptide and GST-tagged protein.¹⁸ An initial 'hit' was selected, and exploratory libraries based on the 'hit' were generated and tested to identify structural features necessary for inhibition of Bfl-1.

The NIH Roadmap Small Molecule Library (at the time, representing approximately 66,000 compounds from BioFocus, San Francisco, CA) was screened for inhibition of GST-Bfl-1. Twenty

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compounds were identified in the initial FP assay to inhibit GST-Bfl-1 with potency of 20 μ M or less. Of those 20 ‘hits’, 14 showed similar GST-Bfl-1 inhibition in the TR-FRET assay. Overall, there was reasonably good agreement between the FP and TR-FRET assay for potency of Bfl-1 inhibition on the basis of IC_{50} values (shown in Tables 1 and 2). The most potent ‘hit’ was compound **1** (Fig. 1) with submicromolar potency in both assays.

‘Hit’ **1** was amenable to structure–activity relationship (SAR) studies. Three regions of the molecule were examined in an iterative process of chemical synthesis and in both FP and TR-FRET in vitro binding assays to define Bfl-1 inhibition SAR potency. The regions for SAR studies included: (1) the chloro substituent(s) and unsaturation of the maleimide ring, (2) substituents on the *N*-aryl ring, and (3) the amine functionality of the maleimide ring (Fig. 1). More than 280 analogs of **1** were prepared to optimize selectivity and potency for inhibition of Bfl-1.

We first investigated the necessity of the chloride substitution and the requirement of unsaturation of the maleimide ring system for potency of Bfl-1 inhibition. Two commercially available analogs of **1** were purchased (ChemBridge, San Diego, CA) and tested as inhibitors of GST-Bfl-1 (Fig. 2). Compound **2** did not have the maleimide chloride substituent but retained the maleimide ring double bond while compound **3** possessed neither the chloride nor the ring double bond. Both compounds were not potent (i.e., $IC_{50} > 10 \mu$ M) in both the FP and TR-FRET assays showing the necessity of each moiety for potent inhibition of GST-Bfl-1.

N-Aryl maleimide analogs were prepared using a convenient two-step process starting from commercially available starting materials (Scheme 1).¹⁹ In the first step, substituted aniline **4** was heated with 1 equiv of dichloromaleic anhydride **5** in acetic acid for 1 h. After extraction and purification by silica gel chromatography, *N*-aryl dichloromaleimide **6** was isolated. Monosubstitution of the dichloromaleimide with amines was done in a medium throughput format. Dichloromaleimides **6** dissolved in dioxane were combined with amines dissolved in tetrahydrofuran at room temperature to afford 3-chloro-1-aryl-4-amino-1*H*-pyrrole-2,5-dione analogs of **7**. Reaction progress was monitored by TLC and reactions were heated if necessary. Products **7** were purified by preparative TLC and verified by electrospray mass spectrometry. Reactions were conducted on a 5 mg scale. Amounts of product isolated ranged from 3 to 5 mg.

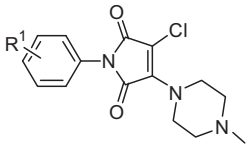
Initially, SAR studies of Bfl-1 inhibition were conducted with compounds of structure **7** where the *N*-methylpiperazine of the side chain amine was kept constant and substituents on the *N*-phenyl ring were varied. A total of 10 anilines (**4**) were used (Table 1).

Because the original ‘hit’ contained 3,4-dichloro substituents in the *N*-aryl ring, maleimides with different permutations of mono- and dichloro-substituted *N*-aryl rings were synthesized. Additional compounds with *N*-aryl methyl, and nitro substituents were prepared to examine possible electronic substituent effects of the *N*-aryl ring on GST-Bfl-1 inhibition. From among compounds **1** and **8–16** (Table 1) the original ‘hit’ **1** was the most potent GST-Bfl-1 inhibitor in both the FP and TR-FRET assays ($IC_{50} = 0.6 \pm 0.1 \mu$ M and $0.8 \pm 0.1 \mu$ M, respectively). Of the dichlorophenyl compounds examined, both the 3,5- (**8**) and 2,4-dichlorophenyl (**9**) substituents afforded IC_{50} values of $2.4 \pm 0.4 \mu$ M and $2.6 \pm 0.2 \mu$ M, respectively, and had similar activities but were threefold less potent than the original ‘hit’ **1**. Other disubstituted dichloro compounds (i.e., **10**) had much lower inhibitory potency (i.e., IC_{50} values of 8.7μ M).²⁰ Compared to compounds **8** and **9**, the 2-chloro or 3-chloro monosubstituted compounds (i.e., **12** and **13**, with IC_{50} values of $3.3 \pm 0.3 \mu$ M and $2.1 \pm 0.2 \mu$ M, respectively) were not significantly different in the inhibition potency of GST-Bfl-1. Monosubstituted *N*-aryl maleimides with either methyl (**14**) or nitro (**15**) groups afforded GST-Bfl-1 inhibitors with much greater IC_{50} values (i.e., $12.6 \pm 2.3 \mu$ M and $6.8 \pm 1.4 \mu$ M, respectively) indicating that alkyl groups or polar groups on the *N*-aryl portion of the maleimide did not increase GST-Bfl-1 inhibition potency. Addition of a 3-methyl group to the 2,6-dichloro aryl group (**11**) further decreased potency 2–3-fold. Pyridine rings in place of the aryl ring also had decreased potency (**16**). Based on these results, the 3,4-dichlorophenyl group was chosen as the optimal substituent of the *N*-phenyl ring for further SAR studies on the piperazine portion of the molecule.

An additional sublibrary of compounds was prepared that retained the 3,4-dichlorophenyl substituent in the *N*-aryl portion of the molecule while the amine group on the maleimide ring was varied (**17–26**). Thus, 3,4-dichloro-1-(3,4-dichlorophenyl)-1*H*-pyrrole-2,5-dione **6** was treated with amines to examine the effect of substitution on the maleimide ring itself. Amines used included morpholines, piperidines, pyrrolidines, cyclohexylamine, ethanolamine, benzylamine, substituted piperazines, piperazinone, and dimethylamine. Dimethylamino **21**, morpholino **22**, and 3-methyl-piperazino **24** afforded compounds equipotent to original hit **1**. The remainder of the amine substituents examined (**17–20**, **23**, and **25–26**) afforded poorly potent compounds. *N*-terminal piperazines with a center of chirality was tested to examine the stereoselectivity of Bfl-1 inhibition (Table 2).

Compounds **24** and **25** have an unsubstituted nitrogen atom but include a methyl group in the α position resulting in a center of

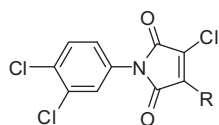
Table 1
Effect of *N*-aryl maleimide ring substitution on GST-Bfl-1 inhibition



| Compound # | R ₁ | Bfl-1 FP ^a IC ₅₀ (μM) | Bfl-1 TR-FRET ^a IC ₅₀ (μM) |
|------------|------------------------------|---|--|
| 1 | 3,4-Dichloro-phenyl | 0.6 ± 0.1 | 0.8 ± 0.1 |
| 8 | 3,5-Dichloro-phenyl | 2.4 ± 0.4 | 1.9 ± 0.2 |
| 9 | 2,4-Dichloro-phenyl | 2.6 ± 0.2 | 1.4 ± 0.1 |
| 10 | 2,3-Dichloro-phenyl | 8.7 ± 1.7 | 2.6 ± 0.1 |
| 11 | 2,6-Dichloro-3-methyl-phenyl | 23.9 ± 3.9 | 11.1 ± 2.3 |
| 12 | 2-Chloro-phenyl | 3.3 ± 0.3 | 1.0 ± 0.1 |
| 13 | 3-Chloro-phenyl | 2.1 ± 0.2 | 1.2 ± 0.1 |
| 14 | 3-Methyl-phenyl | 12.6 ± 2.3 | 10.8 ± 0.9 |
| 15 | 3-Nitro-phenyl | 6.8 ± 1.4 | 3.9 ± 0.4 |
| 16 | 3-Chloro-pyridin-4yl | 29 ± 3.9 | 35.6 ± 3.9 |

^a Data are the mean ± SEM.

Table 2
SAR for analogs of 'hit' **1** with varied amines



| Compound # | R | Bfl-1 FP ^a IC ₅₀ (μM) | Bfl-1 TR-FRET ^a IC ₅₀ (μM) |
|------------|---|---|--|
| 17 | | >50 | >50 |
| 18 | | >50 | >50 |
| 19 | | >50 | >50 |
| 20 | | >50 | >50 |
| 21 | | 0.3 ± 0.2 | 0.4 ± 0.1 |
| 22 | | 0.8 ± 0.1 | 0.45 ± 0.03 |
| 23 | | 1.7 ± 0.2 | 1.0 ± 0.1 |
| 24 | | 0.6 ± 0.3 | 0.8 ± 0.1 |
| 25 | | 2.2 ± 0.4 | 1.1 ± 0.1 |
| 26 | | 5.6 ± 0.4 | 2.0 ± 0.4 |
| 27 | | 10.5 ± 1.7 | 3.3 ± 0.3 |
| 28 | | 0.24 ± 0.03 | 0.62 ± 0.03 |
| 29 | | 0.3 ± 0.2 | 0.40 ± 0.04 |
| 30 | | 4.9 ± 0.8 | 4.1 ± 0.5 |
| 31 | | 9.6 ± 2.0 | 6.2 ± 0.3 |
| 32 | | 1.2 ± 0.4 | 5.0 ± 0.8 |
| 33 | | >10 | 8.3 ± 2.1 |
| 34 | | >10 | >10 |
| 35 | | >10 | >10 |
| 36 | | 2.4 ± 0.2 | 1.3 ± 0.1 |
| 37 | | 4.1 ± 0.4 | 2.1 ± 0.2 |
| 38 | | 0.25 ± 0.05 | 0.69 ± 0.03 |

^a Data are the mean ± SEM.

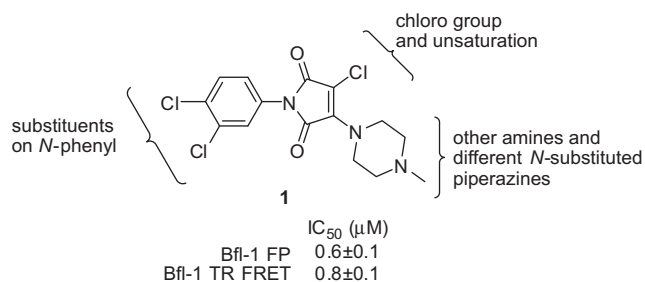


Figure 1. Chemical structure of 'hit' **1** from a high-throughput screen for inhibition of GST-Bfl-1 and regions of 'hit' amenable to SAR work.

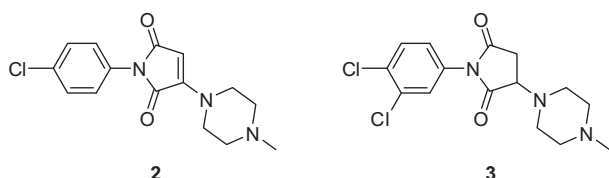
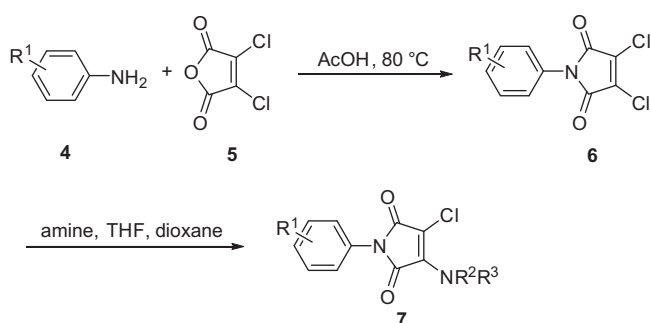


Figure 2. Analogs of **1** without the maleimide chloro atom and double bond moieties necessary for potent GST-Bfl-1 inhibition.



Scheme 1. Synthesis of maleimides following a two-step procedure.

chirality. Compound **24** with *R* stereochemistry at C-3 of piperazine showed considerable Bfl-1 inhibitory potency (i.e., IC₅₀ values for FP and TR-FRET assays of 0.6 ± 0.3 μM and 0.8 ± 0.1 μM, respectively). In contrast, compound **25** with the *S* stereochemistry had considerably less GST-Bfl-1 inhibitory potency (i.e., fourfold greater IC₅₀ value for the FP assay and approximately the same IC₅₀ value for the TR-FRET assay).

Accordingly, another sublibrary of compounds based on compound **1** was prepared that incorporated the 3,4-dichlorophenyl moiety and modification of substituted piperazines to the maleimide structure. Modifications of the 4-*N*-substituent of the piperazine would allow for property adjustment such as solubility, and metabolic stability in further studies. A complete list of 4-*N*-substituents tested is available in the [Supplementary data](#). Compounds **27–38** (Table 2) are the most representatives of the SAR trends for this region of the molecule. *N*-substitution included alkyls, hydroxyl, amine and ether groups, non-substituted and substituted phenyl, benzyl, and cinnamyl groups.

Hydrophilic hydroxy ethyl groups at the terminal piperazine nitrogen atom were associated with the most potent GST-Bfl-1 inhibitory functional activity in this series (i.e., compounds **28** and **29** with IC₅₀ values 0.24 ± 0.03 μM and 0.3 ± 0.2 μM, respec-

tively, from the FP assay). Compared to 'hit' **1**, carbon chains longer than methyl on the piperazine terminal nitrogen decreased potency (i.e., **20** and **21** with 1.7 ± 0.2 μM and 10.5 ± 1.7 μM, respectively). Compared to **1**, *N*-phenylpiperazine (i.e., **24** with IC₅₀ 4.9 ± 0.8 μM) had decreased potency and substitution around the terminal phenyl ring in the *ortho*-, *meta*-, and *para*-positions (i.e., **31**, **33–35** with IC₅₀ > 9 μM) showed lower potency in the FP assay. *Meta*-Methoxy phenyl was the exception **32** showing a moderate potency (i.e., IC₅₀ (FP) value 1.2 ± 0.4 μM) but much improved compared to the *m*-methyl (**33**) and *m*-CF₃ (**34**) equivalents. Both compounds (i.e., **27** and **28**) had IC₅₀ values > 10 μM for GST-Bfl-1 inhibition in the FP assay. The conclusion was that a hydrophilic pocket was present in GST-Bfl-1 or a hydrogen bonding interaction was occurring with GST-Bfl-1 and **32** between the *meta*-position to increase inhibitory potency. Compared with **1**, maleimides with *N*-terminal piperazine benzyl or 3,4-methylenedioxybenzyl substituents (i.e., **35** and **36**, IC₅₀ values of 2.4 ± 0.2 μM and 4.1 ± 0.4 μM, respectively) were less potent GST-Bfl-1 inhibitors by two- and four-fold, respectively. Maleimide **38** with a cinnamyl group on the *N*-terminal piperazine was a potent GST-Bfl-1 inhibitor and comparable to compounds **28** and **29** (i.e., IC₅₀ values of 0.25 ± 0.05 μM vs 0.3 ± 0.2 μM and 0.3 ± 0.2 μM). Data from the TR-FRET assay were similar for all three compounds (0.4–0.69 μM). It is postulated that the BH3 region of Bfl-1 where the amine functionality of the inhibitor resides is large enough to accommodate larger groups on the maleimide ring. A cinnamyl group may induce additional π–π aromatic interactions with Bfl-1 to increased inhibitory potency.

Other combinations of anilines and amines were prepared and tested in order to look at structural synergistic effects (see Table 3 in [Supplementary data](#)): 3-methoxy, 4-nitro, 3-nitro, 4-methyl, 3-trifluoro, 4-trifluoro anilines in combination of the amines used for compounds from Table 2. None of them showed submicromolar potencies.

A potent agent (i.e., **1**) and two less potent agents (i.e., **19** and **21**) were examined in cell-based viability studies or assays using cancer cell lines or other mammalian cell lines.²¹ For compound **1**, inhibition of human H69AR small cell lung tumor cell growth was observed at a concentration of 10 μM. Compound **21** was not inhibitory to cell viability in a mammalian cell line but compound **19** decreased cell viability at 15 μg/mL. Thus, as a class it does not appear that the compounds possess universal toxicity but depending on the structure, certain *N*-aryl maleimide Bfl-1 inhibitors can decrease cancer cell viability or cause toxicity to other mammalian cell lines.

In summary, more than 280 substituted maleimides were prepared in a medium throughput format from readily available starting materials. SAR analysis revealed the effects of substitution on the *N*-phenyl ring and variation of amines on the maleimide ring system, and the necessity of a chloro substituent and a double bond in the maleimide ring for inhibition of GST-Bfl-1. The *N*-3,4-dichloroaryl moiety of the original 'hit' **1** provided the optimal substitution pattern on the *N*-aryl ring. Optimal amines for substitution of one maleimide chloride atom included hydrophilic amines or amines that could participate in hydrogen bonding or π–π interactions. Submicromolar IC₅₀ values for inhibition of Bfl-1 were observed for maleimides substituted with dimethylamine, *N*-methylpiperazine, and piperazines (i.e., **21**, **22**, **24**, **28**, **29**, and **38**) containing water-soluble groups or a cinnamyl group on the terminal nitrogen atom. For one subset of piperazines possessing a center of chirality, considerable stereoselectivity of Bfl-1 inhibition was observed (i.e., **24** > **25**). The SAR studies reported herein provide valuable information for the structural requirements for inhibition of Bfl-1 by maleimides and may provide insight into development candidates for anti-cancer therapeutics.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.046.

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- Peptide Synthesis*: FITC-Bid BH3 peptide was synthesized on the ACT 350 Multiple Peptide Synthesizer using Fmoc chemistry on Wang resin. FITC was coupled to the N-terminus while the peptide was still on the resin. Peptide was cleaved from the resin using 94% TFA/2.5% H₂O/2.5% EDT/1% TIS for 2 h at room temperature. Crude peptide was purified on a Gilson HPLC with 0.1% TFA water/acetonitrile and characterized by mass spectroscopy on an Applied Biosystems Voyager System 6264 using MALDI-TOF ionization. *TR-FRET Assay*: Dose-response curves contained 10 concentrations of compounds obtained using twofold serial dilution. Compounds were serially diluted in 100% DMSO, and then diluted with water to 10% final DMSO concentration. Compounds in 10% DMSO (4 μ L) were transferred into columns 3–22 of Greiner 384-well white small-volume plates (784075). Each compound concentration was assayed in duplicate wells. Columns 1–2 and 23–24 contained 4 μ L of 10% DMSO. Assay buffer (8 μ L of 25 mM Bis-Tris, pH 7.0, 1 mM TCEP, 0.005% Tween 20) was added to columns 1–2, which were reserved for positive controls, using a WellMate bulk dispenser (Matrix). Bfl-1 working solution (8 μ L of 7.4 nM GST-Bfl-1 in assay buffer) was added to columns 3–24 using WellMate bulk dispenser (Matrix). Columns 23–24 represent negative control wells. Plates were incubated for 1 h at +4 °C. Freshly prepared FITC-Bid/Tb-Ab working solution (8 μ L of 5.6 nM FITC-Bid and 2.5 nM Tb-Ab in assay buffer) was added to the whole plate using WellMate bulk dispenser (Matrix). Plates were incubated for 4 h at room temperature protected from direct light. Fluorescence was measured on an M5 plate reader, Molecular Devices (excitation: 340 nm, emission: 490 and 520 nm, cutoff: 475 and 515 nm, respectively) in Time Resolved (TR) mode with signal integrated for 1 ms after initial delay 0.1 ms and averaged from five readings. The TR-FRET signal was calculated as the ratio of TR-Fluorescence at 520 nm to TR-Fluorescence at 490 nm. Data analysis was done using a sigmoidal dose-response equation through non-linear regression. *Statistical Analysis*: Analysis of the inhibition kinetics was done with Graphpad Prism (San Diego, CA) software. A website explaining the meaning of SEM (reported as \pm in the IC₅₀ data in the tables) can be found at: http://graphpad.com/help/prism5/prism5help.html?usingstatisticalanalyses_step_by_s.htm.
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