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14. ABSTRACT

**Purpose:** We are evaluating the efficacy of diclofenac (DFA), an anti-inflammatory agent with L-Selectin sheddase activity, in a murine model of spinal cord injury.

**Scope:** These studies have focused on the efficacy of DFA in the context of dose, optimal therapeutic window, and dependency on injury severity, using clinically relevant outcome measures that include neurologic assessments and assays of bladder function.

**Major findings:**
- We demonstrated that 40 mg/kg DFA is the minimally effective dose to induce L-selectin shedding in a mouse model of spinal cord injury.
- We demonstrated locomotor recovery in mice receiving 40mg/kg DFA up to 3 hours following spinal cord injury.
- We demonstrated improved locomotor recovery using this paradigm for two injury severities, mild and severe, suggesting a robust therapeutic effect.
- We identified no adverse effects to animal health, as evaluated by body weight.
- We identified no added locomotor recovery due to multiple, successive doses of DFA. Moreover, additional doses proved to be toxic and increase animal mortality.

**Significance:** We have identified robust locomotor recovery in both mild and severe spinal cord injured mice that received DFA up to 3 hours following injury. Furthermore, we identified no adverse effects utilizing this dose. Therefore, these promising data suggest that 40mg/kg DFA, administered within 3 hours of spinal cord injury, could be an effective therapeutic intervention for spinal cord injury.

15. SUBJECT TERMS

spinal cord injury, L-Selectin, diclofenac, mouse, urologic function, neurologic function

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INTRODUCTION

This proposal is investigating the hypothesis that the anti-inflammatory drug diclofenac (DFA), acting as an L-selectin sheddase, will improve neurologic outcome and ameliorate neurogenic bladder dysfunction resulting from spinal cord injury (SCI). L-selectin is expressed on the surface of all leukocytes. Preliminary data using the L-selectin knockout (KO) mouse confirmed the dependency of L-selectin on neurologic recovery and thus served as the basis for pharmacologic targeting of this molecule in a murine model of SCI. The specific aims of this proposal are to define the minimal effective dose of DFA, the optimal window of therapeutic intervention for DFA, whether DFA administration improves bladder function, and if the efficacy of DFA is dependent on proteolytic cleavage of L-selectin.

Please note that each task is indicated in bold.

BODY

Specific Aim 1

Task 1. Define the minimal effective dose of DFA

1a. Obtain animal use protocol approval (months 1-4)

We received approval from the UCSF IACUC and ACURO to conduct these studies.

1b. Assay L-Selectin sheddase activity in both blood and spinal cord by flow cytometry at 8 hours to 7 days after a single bolus administration of DFA (at 1, 5, 10, 20, or 40 mg/kg) given immediately after injury (months 5-9)

To assess the L-selectin sheddase activity of increasing doses of DFA, male C57BL/6 mice were subjected to a 2g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level (mild injury). DFA (60mg/kg, 40mg/kg, 20mg/kg, 10mg/kg, 5mg/kg, or 1mg/kg) or vehicle (PBS) was administered immediately following spinal cord injury (SCI). L-selectin sheddase activity was quantified by utilizing an enzyme-linked immunosorbent assay (ELISA) that measures the amount of soluble L-selectin in plasma and spinal cord tissue (n=5/group) collected at 8 hours, 1 day, 3 days, and 7 days following SCI/DFA administration. Data from plasma samples are summarized in Figure 1A-D and data from spinal cord tissue are summarized in Figure 1E-H. Significance for all data was determined using a one-way ANOVA followed by a Dunnett's post-hoc test and was defined as p<0.05.

We identified a significant increase in the levels of soluble L-selectin in the plasma of mice receiving 60mg/kg and 40mg/kg DFA at 8 hours (Figure 1A) and 1 day (Figure 1B) post-injury/administration. By 3 days (Figure 1C), this difference had returned to baseline and continued to be non-significant at 7 days (Figure 1D). The increased levels of soluble L-selectin are indicative of increased L-selectin sheddase activity and suggest that the 60mg/kg and 40mg/kg DFA doses were potent within the first 24 hours of administration. No other doses showed a significant alteration to the soluble levels of L-selectin, suggesting a lack of potency. We also identified a significant increase in the levels of soluble L-selectin in the spinal cords of mice receiving 60mg/kg and 40mg/kg DFA at 8 hours (Figure 1E) and 1 day (Figure 1F) post-injury/administration. As with the plasma data, this difference had returned to baseline at 3 days (Figure 1G) and was still non-significant at 7 days (Figure 1H). These data corroborate the data from the plasma and furthermore indicate that the L-selectin sheddase activity of 60mg/kg and 40mg/kg DFA is occurring at the target site of interest: the injured spinal cord. As with the plasma data, lower doses of DFA exhibited no significant changes to L-selectin levels in the injured spinal cord.

Taken together, these data suggest that the 40mg/kg dose of DFA is the minimum effective dose required for L-selectin sheddase activity.

We next confirmed the shedding activity of 40mg/kg DFA by performing flow cytometry to detect the absence of L-selectin on leukocytes 1 day post-SCI. Male C57BL/6 mice were subjected to a 2g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level (mild injury). DFA (40
mg/kg) or vehicle was administered immediately following spinal cord injury (SCI) (n=5/group). Plasma and spinal cord tissue was collected 1 day post-SCI/DFA administration and processed by flow cytometry. Data from plasmid samples processed by flow cytometry are in Figure 2A, and data from spinal cord samples processed by flow cytometry are in Figure 2B. Significance was defined as p<0.05 and determined using a one-way ANOVA followed by a Dunnett’s post-hoc test for flow data.

Using flow cytometry, we identified a significant reduction in L-selectin expression on circulating leukocytes from plasma at 1 day post-SCI/DFA administration (Figure 2A) and on leukocytes from the spinal cord at 1 day post-SCI/DFA administration (Figure 2B). Taken together, these data suggest that the L-selectin sheddase activity of DFA in the spinal cord can be monitored by utilizing either flow cytometry to observe reductions in L-selectin expression in leukocytes or ELISA to observe increases in soluble L-selectin that has been shed by circulating leukocytes.

In summary, these data demonstrate that 40mg/kg DFA is the minimally effective dose to induce L-selectin shedding from leukocytes following spinal cord injury.

1c. Use a similar dosing strategy and compare neurologic recovery in spinal cord injured mice treated with DFA or vehicle immediately after spinal cord injury (months 10-12).

Male C57BL/6 mice were subjected to a 2g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level. DFA (40mg/kg) or vehicle was administered immediately following SCI (n=15/group), using a randomized, blinded design. Neurologic recovery was measured using the Basso mouse scale (BMS), where 0 indicates complete hind-limb paralysis and 9 indicates normal locomotion. Testing was performed in a blinded fashion weekly 6 weeks post-injury (Figure 3A). A two-way repeated measures ANOVA demonstrated a significant effect for time (p<0.0001), but no effect for treatment (p>0.05). At 1 week post-injury, mice had an average score of ~3, denoting placing of the paw with or without weight support. At 6 weeks, mice had an average score of ~4-5, indicating mice were either occasionally plantar stepping or frequently/consistently plantar stepping.

A model of mild injury severity is challenging as there is risk of a ceiling effect, whereby treatment will exert no benefit. That is, as animals quickly reach a ceiling of recovery and plateau, the ability to detect drug-dependent effects on locomotor recovery is reduced. Equally importantly, the BMS is an ordinal scale. As such, differences between grades are not linear and in certain cases fail to identify clinically relevant changes. For example, a score of 4 delineates occasional plantar stepping whereas a score of 5 is assigned to frequent/consistent plantar steppers. Such a change in mobility has strong clinical relevance that is not captured by this unweighted scoring system. Given these limitations, we sought to classify mice based on the ability to frequently/consistently plantar step as a method of assessing this portion of the BMS scale (Figure 3B). Frequent/consistent plantar stepping was observed in 40% of vehicle mice vs. 92% of 40mg/kg DFA mice. Chi square frequency analysis with fisher’s exact test demonstrated a significant effect between mice receiving vehicle and 40mg/kg DFA (p<0.05). Thus, mice receiving 40mg/kg DFA had improved plantar stepping consistency.

To further investigate locomotor recovery, at 6 weeks post-injury, mice were tested for coordination by walking across a grid for 3 minutes. One mouse from the vehicle group was excluded due to the inability to step. The number of foot faults, indicative of a lack of coordination, was normalized to the total distance travelled by the mouse (Figure 3C). A Kruskal-Wallis test demonstrated a significant reduction in errors made by mice receiving 40mg/kg DFA (p<0.05). These data suggest that DFA administration was improving the overall coordination of mice in following mild SCI.

Together, these data demonstrate that mice with mild SCI and receiving 40mg/kg DFA showed significant locomotor recovery relative to the vehicle controls.

Mice were weighed prior to injury, 1 and 3 days post-injury, and then weekly thereafter (Figure 4A). A two-way repeated measures ANOVA demonstrated a significant effect for time (p<0.0001), but no effect for treatment (p>0.05). Notably, mice lost weight shortly after SCI, then regained this weight, ultimately ending with a higher weight than prior to surgery due to aging. These data demonstrate that DFA administration did not adversely affect animal weight and overall health in a mild-SCI paradigm.
Next, we sought to evaluate bladder function in DFA-treated mice by awake cystometry in a subset of mice (n=9/group). Consistent with this being a mild injury paradigm, bladder functional recovery may have reached a ceiling effect, similar to the observations of locomotor recovery. When cystometry was performed at the end of the study, 29/34 (~85%) of mice exhibited partial or complete voluntary bladder voiding during daily animal care. Following euthanasia, bladders were removed, weighed, and normalized to total bodyweight (Figure 4B). A one-tailed t-test demonstrated no significant differences on normalized bladder weights between groups (p>0.05). Following SCI, bladder dyssynergia typically results in alterations to the levels of smooth/skeletal muscle, as well as connective tissue matrices, leading to a thickening of the bladder wall. However, all SCI mice in this study, regardless of treatment, demonstrated normalized bladder weights comparable to historical uninjured controls. Therefore, any potential DFA-dependent effects on bladder recovery may have been obscured by this degree of baseline recovery. The volume of residual urine (Figure 4C) and the number of uninhibited bladder contractions/voiding cycle (Figure 4D) for all mice have been calculated, and the remaining analysis of cystometry data is currently in progress. A Kruskal-Wallis test for each measure demonstrated no significant differences for the volume of residual urine or number of uninhibited bladder contractions/voiding cycle between groups (p>0.05). Taken together, these data suggest that urologic function is not being altered by DFA in this mild injury paradigm. However, the ability to detect DFA-dependent effects on urological function could be increased in a more severe SCI paradigm that has a wider range of recovery.

1d. Conduct morphometric analyses (spared white matter, glial scarring, serotonergic fiber tracts) of the cords, prepared from animals in 1c (months 13-15).

We collected spinal cords and bladders from mice in 1c and have stored them frozen. We plan to perform all histological evaluations of this tissue in tandem with tissue from Task 2 to minimize variability and increase efficiency.

**Specific Aim 2**

**Task 2. Determine the optimal window of therapeutic intervention for DFA.**

2c. Determine if optimal dosing of DFA, defined in 2b, supports neurologic recovery after a more severe spinal cord injury (months 24-26).

Due to the challenges in assessing locomotor recovery with a mild SCI (discussed above), we elected to move to a more severe SCI model prior to performing tasks 2a and 2b. Critically, mice receiving a severe SCI lose the ability to perform weight supported steps, providing an ideal model to assess locomotor recovery.

Male C57BL/6 mice were subjected to a 3g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level. DFA (40mg/kg) or vehicle was administered immediately following SCI (n=15/group), using a randomized, blinded design.

Neurologic recovery was measured using the Basso mouse scale (BMS), where 0 indicates complete hind-limb paralysis and 9 indicates normal locomotion. Testing was performed in a blinded fashion 3 hours, 1 day, 3 days, and then weekly for 6 weeks post-injury (Figure 5A). A two-way repeated measures ANOVA demonstrated a significant effect for time (p<0.0001) and for treatment (p<0.0001). A Sidak’s multiple comparisons test demonstrated that mice with severe SCI that received 40mg/kg DFA immediately after injury exhibited improved locomotor recovery starting at 3 days post-SCI (p<0.05) and persisting until 6 weeks (p<0.0001).

We next sought to classify mice based on the ability to step as a method of assessing recovery (Figure 5B). Stepping was observed in 8% of vehicle mice vs. 69% of 40mg/kg DFA mice. Chi square frequency analysis with fisher’s exact test demonstrated a significant effect between mice receiving vehicle and 40mg/kg DFA (p<0.01). Thus, mice receiving 40mg/kg DFA had improved ability at stepping.

Mice were weighed prior to injury, 1 and 3 days post-injury, and then weekly thereafter (Figure 5C). A two-way repeated measures ANOVA demonstrated a significant effect for time (p<0.0001), but no effect for treatment (p>0.05). Notably, mice lost weight shortly after SCI, and ultimately never regained full weight. This is likely due to the severity of the injury affecting muscle tone. Following euthanasia, bladders were removed, weighed, and normalized to total bodyweight (Figure 5D). A one-tailed t-test demonstrated no significant differences on normalized bladder weights between groups (p>0.05). These data demonstrate that DFA
administration did not adversely affect animal weight and overall health in a severe SCI paradigm. Furthermore, bladder thickness and weight did not appear to be influenced by DFA.

Taken together, these data demonstrated that administration of 40mg/kg DFA immediately after a severe SCI supports locomotor recovery.

2a. Evaluate long-term neurologic recovery in mice after a moderate level of spinal cord injury that have been treated with a bolus injection of DFA or vehicle at 3 hours. If there is benefit, then repeat study but initiate treatment at 8 hours. If there is no benefit at 3 hours, then repeat study with DFA/vehicle given at 1 hour (months 16-20).

Given the success with the more severe model of SCI in 2c (discussed above), we elected to continue using this model for the remaining aims as it represents a better model at differentiating locomotor recovery and is more clinically relevant. We next evaluated the efficacy of DFA administration 3 hours following a severe SCI. Male C57BL/6 mice were subjected to a 3g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level. DFA (40mg/kg) or vehicle was administered 3 hours following SCI (n=15/group), using a randomized, blinded design.

Neurologic recovery was measured using the Basso mouse scale (BMS), where 0 indicates complete hind-limb paralysis and 9 indicates normal locomotion. Testing was performed in a blinded fashion 3 hours, 1 day, 3 days, and then weekly for 6 weeks post-injury (Figure 6A). A two-way repeated measures ANOVA demonstrated a significant effect for time (p<0.0001) and for treatment (p<0.001). A Sidak’s multiple comparisons test demonstrated that mice with severe SCI that received 40mg/kg DFA immediately after injury exhibited improved locomotor recovery starting at 1 week post-SCI (p<0.05) and persisting until 6 weeks (p<0.001).

We next sought to classify mice based on the ability to step as a method of assessing recovery (Figure 6B). Stepping was observed in 18% of vehicle mice vs. 62% of 40mg/kg DFA mice. Chi square frequency analysis with fisher’s exact test demonstrated a significant effect between mice receiving vehicle and 40mg/kg DFA (p<0.05). Thus, mice receiving 40mg/kg DFA had improved ability at stepping.

Taken together, these data demonstrated that administration of 40mg/kg DFA 3 hours after a severe SCI supports locomotor recovery.

Accordingly, we next evaluated locomotor recovery in mice that received DFA 8 hours following a severe SCI. Male C57BL/6 mice were subjected to a 3g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level. DFA (40mg/kg) or vehicle was administered 8 hours following SCI (n=15/group), using a randomized, blinded design.

Neurologic recovery was measured using the Basso mouse scale (BMS), where 0 indicates complete hind-limb paralysis and 9 indicates normal locomotion. Testing was performed in a blinded fashion 3 hours, 1 day, 3 days, and then weekly for 6 weeks post-injury (Figure 7A). A two-way repeated measures ANOVA demonstrated a significant effect for time (p<0.0001) and no effect for treatment (p>0.05), demonstrating that mice that received DFA 8 hours post-severe SCI did not exhibit locomotor recovery.

We next sought to classify mice based on the ability to step as a method of assessing recovery (Figure 7B). Stepping was observed in 15% of vehicle mice vs. 36% of 40mg/kg DFA mice. Chi square frequency analysis with fisher’s exact test demonstrated a no significant effect between mice receiving vehicle and 40mg/kg DFA (p>0.05). Thus, mice receiving 40mg/kg DFA 8 hours post-SCI did not exhibit improved ability at stepping.

Taken together, these data demonstrated that administration of 40mg/kg DFA 8 hours after a severe SCI does not support locomotor recovery. Therefore, the optimal therapeutic window for efficacy is within a time frame of less than 8 hours post injury. Thus, while efficacy is clearly evident when treatment is initiated at 3 hours of injury, our findings do not rule out the possibility that DFA given after 3 hours but before 8 hours might likewise prove beneficial. Such a distinction becomes important given the challenges in the field where accessibility to treatment may be delayed.

2b. Assess neurologic recovery using multiple doses of DFA, as defined in 2a. Compare mice that are given DFA 1 day after the first dose, 1 and 2 days after the first dose, and 1,2, and 3 days after the first dose (months 21-22).
We next sought to define whether multiple doses of 40mg/kg DFA, administered within the therapeutic window at 3 hours and successively for up to 3 days. Male C57BL/6 mice were subjected to a 3g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level. DFA (40mg/kg) or vehicle was administered 3 hours following SCI, and then a subset of DFA-treated mice received either one 40mg/kg DFA dose 24 hours post-SCI or two 40mg/kg DFA doses at 24 and 48 hours post-SCI (n=15/group), using a randomized, blinded design.

As we performed the study, we observed high mortality in certain animals. Experimenters, not blinded to groups, determined that mortality occurred primarily in mice receiving multiple doses of DFA (Figure 8A). 100% of mice that received 3 total doses of DFA (3 hours, 1 day, and 3 days post-SCI) died. 47% of mice that received 2 total doses of DFA (3 hours and 1 day post-SCI) died. However, vehicle and single dose DFA-treated mice at 3 hours did not exhibit increased mortality outside normal levels (~10-15%). These data suggest multiple doses of 40mg/kg DFA are toxic. We elected to continue assessing behavior in the remaining mice as a replication of the previous data and to observe if the surviving subset of mice (n=7) exhibited locomotor recovery.

Neurologic recovery was measured using the Basso mouse scale (BMS), where 0 indicates complete hind-limb paralysis and 9 indicates normal locomotion. Testing was performed in a blinded fashion 3 hours, 1 day, 3 days, and then weekly for 6 weeks post-injury (Figure 8B). A two-way repeated measures ANOVA demonstrated a significant effect for time (p<0.0001) and for treatment (p<0.01). A Sidak’s multiple comparisons test demonstrated that mice with severe SCI that all 40mg/kg DFA treated groups (single and double doses) exhibited locomotor recovery starting at 1 week post-SCI (p<0.001 for single and p<0.01 for double) and persisting until 6 weeks (p<0.0001 for single and p<0.05 for double). Critically, there were no significant differences (p>0.05) between single and double-dose DFA groups, indicating no conferred benefit to additional doses.

Taken together, these data demonstrated that administration of multiple doses of 40mg/kg DFA after a severe SCI do not confer any added benefits over a single dose of 40mg/kg DFA. Moreover, multiple doses increase the risk of toxicity and adverse side-effects, indicating a single dose regimen of 40mg/kg DFA is most appropriate clinically.

We finally sought to test for the development of pain syndromes in mice post-severe SCI and whether DFA attenuates this process (Figure 8C). We utilized the tail-flick test, which administers a non-noxious heat stimulus to the tail of mice and determines the time to withdrawal. Mice were tested pre-injury and at 6 weeks post-injury by experimenters blinded to treatment groups. We observed no significant differences between vehicle and DFA-treated mice (p>0.05). However, we also detected no significant difference between pre-injury and post-injury responses (p>0.05), suggesting a lack of development of a pain syndrome. Therefore, we can conclude that DFA treatment post-severe SCI does not appear to exacerbate pain, but a more robust model that develops pain would be needed to assess any therapeutic efficacy of DFA for thermal hyperalgesia.

**Specific Aim 3**

**Task 3. Determine if DFA improves bladder function**

3a. Using optimal dosing defined in 2c, compare urologic function in spinal cord injured mice treated with either vehicle or DFA. (months 27-29).

We performed awake cystometry on vehicle and 40mg/kg DFA treated mice 3 hours post-severe SCI from aim 2b (see above) 7 weeks post-SCI. Analysis of these data is currently underway and expected to be completed in the coming months.

**Specific Aim 4**

**Task 4. Determine if efficacy of DFA is dependent on its proteolytic cleavage of L-selectin.**

The L(E) same mice for Specific Aim 4 were provided by a collaborator at another institution and were housed under conditions that did not meet the requirements necessary for importation into the mouse barrier facility at UCSF. As such we have been required to first rederive these animals before they can be housed in the UCSF
barrier facility. We are in receipt of breeding pairs and have begun the rederivation process. The mice have been successfully rederived and are currently awaiting transfer to our housing room. We plan to begin breeding these mice to sufficient numbers for the experiments in Specific Aim 4 in the coming months.

Key Research Accomplishments

- Identified 40mg/kg DFA as minimal effective dose required for L-selectin sheddase activity
- Demonstrated immediate administration of 40mg/kg following mild SCI results in improved locomotor recovery
- Demonstrated that 40mg/kg DFA does not have adverse effects on animal health
- Demonstrated efficacy of 40mg/kg DFA is retained following severe SCI
- Demonstrated optimal therapeutic window for 40/mg/kg DFA is up to at least 3 hours post-SCI (and possibly longer). However, no benefit is seen when treatment is initiated at 8 hours post – SCI.
- Demonstrated no benefit to multiple doses of DFA

Conclusions

- 40mg/kg DFA is the minimal effective dose to induce L-selectin shedding in the plasma and spinal cord following SCI
- 40mg/kg DFA improves locomotor recovery in both mild and severe SCI when administered within 3 hours and has no adverse effects
- DFA does not improve, nor exacerbate, bladder recovery or pain following SCI
Figure 1: 60mg/kg and 40mg/kg DFA induce shedding of L-selectin from leukocytes in plasma and spinal cord following spinal cord injury as detected by ELISA
Figure 2: Flow cytometry confirms shedding of L-selectin from leukocytes in the plasma and spinal cord by 40mg/kg DFA following spinal cord injury.
Figure 3: 40mg/kg DFA administered immediately post-mild SCI improves locomotor recovery
Figure 4: 40mg/kg DFA administration post-mild SCI does not influence animal weight, bladder weight, or measures of bladder function.
Figure 5: 40mg/kg DFA administration immediately post-severe SCI improves locomotor recovery and does not influence animal weight or bladder weight.
Figure 6: 40mg/kg DFA administration 3 hours post-severe SCI improves locomotor recovery
Figure 7: 40mg/kg DFA administration 8 hours post-severe SCI does not improve locomotor recovery
Figure 8: Multiple doses of 40mg/kg DFA administered beginning 3 hours post-severe SCI are toxic and do not confer benefits to locomotor recovery over a single dose.
Targeting L-Selectin to Improve Neurologic and Urologic Function After Spinal Cord Injury

Study/Product Aim(s)

- Specific Aim 1: Define minimal effective dose of DFA
  - We defined the minimally effective dose as 40mg/kg DFA based on sheddase activity measured by ELISA and flow cytometry and demonstrated locomotor recovery in spinal cord injured mice
  - Specific Aim 2: Determine the optimal window of therapeutic intervention for DFA
  - We defined the optimal regimen for DFA as a single-dose given 3 hours following spinal cord injury and demonstrated that this regimen is effective for a more severe spinal cord injury

Approach

ELISAs were utilized to identify 40mg/kg as the minimal effective dose of DFA that induced L-selectin shedding in plasma and spinal cord. Neurologic recovery (BMS) was performed on spinal cord injured mice that received either saline or 40mg/kg DFA immediately, 3 hours, or 8 hours post-injury in a mild or severe model of spinal cord injury.

Timeline and Cost

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