

## Title Page

# Inhibition of Ribonucleotide Reductase Reduces Neointimal Formation Following Balloon Injury

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Abbreviations: PTCA, percutaneous transluminal coronary angioplasty; RR, Ribonucleotide Reductase; VSMC, vascular smooth muscle cells; SMC, smooth muscle cells; dNTP, deoxynucleotide triphosphate; HU, hydroxyurea; PBS, phosphate buffered saline; PDGF, platelet derived growth factor; NFκB, nuclear factor κB; NO, nitric oxide.

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## Abstract

Percutaneous transluminal coronary angioplasty (PTCA) has greatly benefited patients with occluded coronary arteries, but its benefits have been undermined by a high incidence of restenosis. The introduction of coronary stents has significantly improved the short and long term outcome but restenosis still occurs in approximately 15-30 % of patients within 6 months. Research efforts are now being directed toward combination stenting and drug delivery. Among the therapeutic targets being pursued are agents which can impede smooth muscle cell migration and proliferation as these processes are critical components of restenosis injury. We propose that inhibiting the conversion of ribonucleotides to deoxyribonucleotides will impede cell proliferation and as such limit the degree of restenosis. Therefore, we tested whether the potent Ribonucleotide Reductase inhibitors, Didox and Imidate, can limit the neointimal proliferation associated with restenosis using a rat carotid model of balloon dilatation injury. Results demonstrated that both Didox and Imidate significantly reduced intimal thickening, resulting in a 71% and 62% decrease in the intima/media ratio, respectively. Similar efficacy was seen with the commercially available RR inhibitor, Hydroxyurea, demonstrating the importance of this enzyme in vascular remodeling. Results from cell proliferation studies suggest that the mechanism of protection is inhibition of SMC proliferation. In addition, Didox and Imidate (100 micromM) are potent inhibitors of SMC migration, which may also contribute to their vascular protective effects. These results suggest that inhibition of Ribonucleotide Reductase may provide a potent strategy to prevent post PTCA restenosis.

## Introduction

The use of percutaneous transluminal coronary angioplasty (PTCA) has greatly reduced the number of fatalities in patients who suffer myocardial infarction (Fischman et al., 1994; Elezi et al., 1998; Bennett and O'Sullivan, 2001). During PTCA, the artery walls are expanded by several times their original diameter in an attempt to increase lumen diameter and improve flow. Unfortunately, this technique is plagued by a high incidence of vessel renarrowing or restenosis occurring in 30-40% of patients within six months of the procedure (Anderson et al., 1993; Fischman et al., 1994; Elezi et al., 1998; Bennett and O'Sullivan, 2001; Heckenkamp et al., 2002). Prevention of restenosis after successful PTCA remains one of the most challenging tasks in the treatment of obstructive coronary artery disease. Attempts to ameliorate this proliferative response involve the use coronary stents, which have significantly improved both short term and long term outcome following interventional coronary revascularization procedures. Despite a reduction in restenosis rate with stent deployment, restenosis still occurs in 15-30 % of patients within 6 months (Fischman et al., 1994; Elezi et al., 1998). This incidence of in-stent restenosis is expected to increase as coronary stenting is becoming more frequent and is used in less ideal lesions. Therefore, in addition to mechanical intervention, pharmacological approaches to reduce the incidence and degree of restenosis are needed.

The vascular trauma associated with PTCA involves a cascade of molecular and cellular events occurring within the vessel wall involving the release of a variety of vasoactive, thrombogenic, and mitogenic factors (Bauters and Isner, 1997; Libby and Tanaka, 1997; Goldschmidt-Clermont and Moldovan, 1999). Within this cascade, several mechanisms contribute to restenosis including elastic recoil, thrombosis, smooth muscle cell migration/proliferation and matrix formation. The result of these vascular events is intimal

hyperplasia, whereby vascular smooth muscle cells (VSMC's) migrate from the media to the intima, proliferate, and consequently form the neointima. During this proliferative response, SMCs undergo a phenotypic modulation from a contractile to a synthetic phenotype (differentiation) (Epstein et al., 1991; Noda-Heiny and Sobel, 1995; Ueda et al., 1995; Farb et al., 2002; Indolfi et al., 2003). Because ultimately, the cascade of events following vascular trauma culminates in cell proliferation and neointimal hyperplasia, it would follow then that among targets being pursued would be agents which can impede smooth muscle cell migration and proliferation, as these processes are critical components of restenosis injury.

In this regard, we have tested the ability of two ribonucleotide reductase (RR) inhibitors to limit the degree of restenosis following balloon mediated dilatation injury in the rat. RR catalyzes the reductive conversion of ribonucleotides to deoxynucleotides. This reductive reaction is a prime target for impeding cellular proliferation, and therefore amenable to inhibiting VSMC replication, because it is a rate limiting step in the biochemical pathway leading to DNA synthesis and thus cell replication (Elford et al., 1970; Elford et al., 1979; Takeda and Weber, 1981; Natsumeda et al., 1985; Anderson et al., 1993; Tanaka et al., 2000). DNA synthesis cannot occur without invoking this reaction since the endogenous pools of dNTP in mammalian cells are inadequate to support new DNA synthesis (Elford et al., 1970; Elford et al., 1979).

Therefore, in the present study we have examined the ability of the polyhydroxy-phenolic compounds, Didox (3,4-dihydroxybenzohydroxamic acid) and Imidate (3,4,5-hydroxybenzimidate), to limit the degree of restenosis following vascular injury. Treatment with either Didox or Imidate resulted in > 60% reduction in neointimal area. *In-vitro* studies

demonstrated that these effects are mediated through both a reduction in SMC proliferation and migration. These results suggest that inhibition of RR may serve as a novel therapeutic target in the treatment of vasculoproliferative disorders such as restenosis.

## Methods

### Materials

Didox, Imidate and Hydroxyurea were provided by Molecules for Health Inc. (Richmond, VA). Rat vascular smooth muscle cells and culture media were purchased from ATCC (Manassas, VA). Fogarty embolectomy catheters were purchased from M & I medical (Miami, FL). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

### Carotid Injury

Male Wistar rats, weighing 400-450 g (Harlan) were fed standard pellet feed and given water ad libitum. The experimental protocol was designed in accordance with Institutional ILACUC standards. Animals were anesthetized with Isoflurane (1.5-2%) in air. The right carotid artery was exposed and a 2F Fogarty balloon embolectomy catheter (Baxter) was inserted via an external carotid arteriotomy incision. The catheter was advanced to the common carotid artery, inflated to a pressure of 2 atm. and rotated in a forward and retrograde direction. The catheter was then deflated and the process repeated three times. Treatment cohorts were divided into 5 groups (n=6-8/group): Control (sham operated), Didox ( $200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ), Imidate ( $200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ), HU ( $200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ) and vehicle (saline). Drugs were administered daily by i.p. injection for a period of 7 days after injury. At 2 weeks post-injury, rats were euthanized by pentobarbital overdose and perfused with 10% buffered formalin. Carotid arteries were removed and placed in the same fixative. Tissues were then embedded in

paraffin, and 4 to 5 sections (4  $\mu\text{m}$ ) were cut at multiple levels. These sections were then stained with hematoxylin-eosin or elastic-van Gieson stain. Sections were examined microscopically and the cross-sectional areas of the lumen, neointima (from the internal elastic lamina to the lumen), and media were determined using digital microscopy with Spot Advanced software. The intima-to-media (I/M) ratio was then calculated from the determined mean. The data represent the mean  $\pm$  SD.

### **Flow cytometry**

SMC were plated on 6 well dishes at a density of  $2 \times 10^5$  cells per well. Cells were then treated with Didox (0-200  $\mu\text{M}$ ), Imidate (0-200  $\mu\text{M}$ ) or HU (0-1000  $\mu\text{M}$ ) and incubated for 24 hours. Following the 24 hour incubation period, the cells were trypsinized and collected in 15 mL centrifuge tubes. The cells were then centrifuged 5 minutes at 800 x g. The supernatant was discarded and the pellet was resuspended in 5 ml PBS. The cells were centrifuged 6 minutes at 200 x g. The supernatant was removed and the pellet was then thoroughly resuspended in 0.5 ml PBS. The cell suspension was transferred into tubes containing 70% ethanol, keeping the cells in fixative >2 hours. The ethanol suspended cells were centrifuged 5 minutes at 200 x g, and the ethanol was decanted. The cell pellet was resuspended in 5 ml PBS and after one minute was centrifuged 5 min at 200 x g. The top layer of liquid was again removed and the cell pellet was resuspended in 1ml PI/Triton X-100 staining solution with RNase A. This staining solution was then incubated at room temperature for 30 minutes. Flow cytometry was then performed using a FACS Calibur (Becton Dickenson). The data are presented as the mean.

### **Intracellular dNTP quantitation**

SMC were plated in T150 flasks and treated with Didox (0-200  $\mu$ M), Imidate (0-200  $\mu$ M) or HU (0-1000  $\mu$ M) and incubated for 24 hours. Following the 24 hour incubation period, the cells were trypsinized and collected in 50 mL centrifuge tubes along with the incubation media. All the extraction steps were performed on ice. Immediately before processing, cells were counted and viability determined using the trypan blue exclusion method. The cells were then centrifuged 5 minutes at 800 x g. The cell pellet was then deproteinized with the same volume of 6% TCA, vortexed for 20s and incubated on ice for 10 minutes. The acid cell extracts were centrifuged 10 min at 2000 x g. The supernatant supernatants were then supplemented with an equal volume of distilled water, vortexed for 60 s and neutralized by the addition of 5M  $K_2CO_3$  prior to HPLC analysis. dNTP detection was carried out using an ESA (Chelmsford, MA) HPLC chromatographic system with UV-Vis detection. Chromatographic separations were performed using a TosoHaas C18 reverse phase column (ODS 80Tm 250 X 4.6 mm, 5 $\mu$ m pore). The mobile phase was delivered at a rate of 1.0 mL/min during the analysis using the following stepwise gradient elution program: A-B (80-20) at 0 min; (40-60) at 30 min; (40-60) at 40 min and (80-20) at 45 min. Buffer A contained 10 mM tetrabutylammonium sulfate, 10mM  $KH_2PO_4$  and 0.25% MeOH, and adjusted to pH 6.9. Buffer B consisted of 5.6 mM tetrabutylammonium sulfate, 50 mM  $KH_2PO_4$  and 30% MeOH, and neutralized to pH 7.0. The injection volume for analysis was 50 $\mu$ L. Detection was carried out at 254 nm.

### **Smooth muscle cell migration**

A wound scrape assay was performed using rat vascular SMC. The cells were grown to confluence on 60 mm dishes. The cells were then made quiescent by incubating them in media containing 0.1% serum for 24 hrs followed by treatment with Didox, Imidate (0-100  $\mu$ M) or



HU (0-1000  $\mu$ M) in media containing 0.1% serum and platelet derived growth factor (PDGF) at concentration of 10 ng/mL. Linear wounds were made by scraping each plate with the tip of a 20  $\mu$ L pipette. The ability of cells to migrate across the wound area was measured using digital microscopy. SPOT advanced software was used to measure the wound immediately, 2, 4, 12 and 24 hrs following injury. The data are presented as rate of migration and represent the mean  $\pm$  SD.

## Results

### **Effects of Ribonucleotide Reductase Inhibitors on Restenosis Following Vascular Injury**

The effects of Didox, Imidate and Hydroxyurea on the vascular remodeling process following arterial injury were determined using a rat model of balloon mediated carotid injury. Each compound (200 mg/kg/day) was delivered via i.p. injection for a period of 7 days post injury. These dosages are based on previously published reports and represent  $\frac{1}{2}$  the maximum tolerated dose in rats (Gupta and Yaffe, 1982; Vaughan et al., 1989). Furthermore, we and others have demonstrated that these doses are sufficient to inhibit RR activity without causing significant toxicity (Mayhew et al., 1999; Mayhew et al., 2002; Horvath et al., 2004).

At 14 days post injury the animals were sacrificed and morphometric analysis was carried out in order to assess the histopathological changes in the vessel wall (fig. 1). Didox treatment resulted in a 62% decrease in neointimal area and a 61% decrease in intima/media ratio (fig. 2). Imidate treated groups demonstrated a 57% decrease in neointimal area and a 55% decrease in the intima/media ratio (fig. 2). Because Didox and Imidate possess other chemical attributes in addition to their effects on RR (Fritzer-Szekeres et al., 1997; Lee et al., 1997; Fritzer-Szekeres

et al., 2000; Fritzer-Szekeres et al., 2002; Inayat et al., 2002; Shet et al., 2003)the commercially available RR inhibitor, HU (200mg/kg/day), was also tested. HU afforded similar vascular protective effects to those observed with Didox and Imidate, resulting in a 55% decrease in neointimal area and a 63% decrease in intima/media ratio (fig. 2). These results suggest that RR inhibition can modulate the remodeling process following vascular injury. However, because the remodeling process occurs over a prolonged period of time, additional studies were performed in order to determine whether the vascular protective effects observed would be mitigated over time.

To further investigate long-term efficacy of the one week dosing regimen, we increased the duration of the study period to 6 weeks. We found that the degree of neointimal thickening at 6 weeks post-injury was increased by 57% as compared to the 2 week study paradigm (fig. 3). Interestingly, the beneficial effect of RR inhibition persisted over the long term. Each compound (200 mg/kg/day) was given ip for a period of 7 days followed by a 5 week recovery period. At the end of the 6 week period, the Didox treated group exhibited a 64% decrease in neointimal area and a 71% decrease in intima/media ratio (fig 3). Similarly, Imidate offered a 61% reduction in neointimal area and a 62% reduction in intima/media ratio (fig 3). HU treatment reduced neointimal formation by 71% and decreased the intima/media ratio by 75% (fig. 3). These results suggest that activation of RR is an early trigger in the vascular response to injury and that inhibition of this enzyme can limit the neointimal proliferation associated with restenosis.

#### **Effects of Didox, Imidate and HU on SMC proliferation.**

Didox, Imidate and HU are known to be potent RR inhibitors (Elford, 1968; Elford et al., 1970; Elford et al., 1979). RR catalyzes the reductive conversion of ribonucleotides to

deoxynucleotides. This reductive reaction is a prime target for impeding cellular proliferation, and therefore amenable to inhibiting VSMC replication, because it is a rate limiting step in the biochemical pathway leading to DNA synthesis (Elford et al., 1970; Elford et al., 1979; Takeda and Weber, 1981; Natsumeda et al., 1985). The ability of Didox and Imidate to inhibit RR activity has been well documented with published reports demonstrating an  $IC_{50}$  of 3-30  $\mu M$  for this class of compounds (Elford et al., 1979). These values represent a > 10 fold increased effectiveness over the classical RR inhibitor HU (Elford, 1968). Based on this evidence, experiments were performed in order to determine whether the ability of these compounds to reduce neointimal formation was due to their ability to inhibit SMC proliferation *in-vitro*. Therefore, we determined the  $IC_{50}$  of each compound on inhibition of smooth muscle cell growth. Cells were incubated in the presence of Didox (0-200  $\mu M$ ), Imidate (0-200  $\mu M$ ) and HU (0-1000  $\mu M$ ) for 24 hours. Cell numbers were then counted using flow cytometry and the concentration at which cell division was inhibited by 50 % ( $IC_{50}$ ) was calculated. Didox yielded an  $IC_{50}$  of 67  $\mu M$ , Imidate was slightly less potent exhibiting an  $IC_{50}$  of 82  $\mu M$  while HU was the least potent with an  $IC_{50}$  of 266  $\mu M$  (fig. 4). These results are consistent with RR activity data and demonstrate that Didox and Imidate are 3-4 times more potent than HU at arresting cell division.

In order to further validate that the observed anti-proliferative properties afforded by these compounds was through inhibition of RR activity, we measured the effects of these compounds on intracellular dATP pools. Results demonstrated that Didox (0-200  $\mu M$ ), Imidate (0-200 $\mu M$ ) and HU (0-1000  $\mu M$ ) dose dependently depleted the endogenous dNTP pools with maximal reductions in dATP content of 58%, 42% and 69%, respectively (Table 1.).

The concentrations at which these drugs afford their *in-vitro* biological effects are well below the range of the peak plasma levels (300-400  $\mu\text{M}$ ) measured following Didox and Imidate infusion (200 mg/kg/day) and would be expected to inhibit RR activity based on the published and observed  $K_i$ 's for these compounds (Elford et al., 1979). These results would suggest that part of the vascular protective effects of these compounds is due to their ability to impede SMC proliferation.

### **Effects of Didox, Imidate and HU on SMC migration.**

However, SMC migration is also a critical component of neointimal proliferation. Therefore, additional studies were performed in order to determine the effects of these compounds on SMC proliferation. Using a wound scrape assay, SMC migration studies were carried out in the presence of Didox, Imidate and HU. VSMC's were cultured to confluence on 60 mm dishes. The cells were made quiescent by incubating in media containing 0.1 % serum. Following 24 hours of serum deprivation, Didox, Imidate and HU were added to the wells (10-1000 $\mu\text{M}$ ) in the presence of PDGF (10 ng/mL) and a linear wound was made across the plate. SMC migration across the wound was monitored by digital microscopy over a 24 hour period. Results from these studies demonstrated that Didox (100  $\mu\text{M}$ ) and Imidate (100  $\mu\text{M}$ ) treatment almost completely inhibited SMC migration, decreasing the migratory rate from 15.8  $\mu\text{M}/\text{hr}$  in the control to 1.7  $\mu\text{M}/\text{hr}$  and 0.9  $\mu\text{M}/\text{hr}$ , respectively (fig. 5). In contrast, HU (100  $\mu\text{M}$ ) had little effect on SMC migration, resulting in a migratory rate of 15.1  $\mu\text{M}/\text{hr}$  (fig. 4). No further inhibition was seen with HU concentrations up to 1 mM. These results demonstrate that Didox and Imidate significantly impair SMC migration. This would be expected to contribute to the

vascular protective effects afforded by these drugs. However, RR inhibition appears to be the principal mechanism as the rat arterial injury data demonstrated similar efficacy with HU.

## Discussion

Although PTCA and coronary artery stenting have had a tremendous impact on the treatment of coronary vascular disease, these procedures are marked by a high incidence of restenosis (Anderson et al., 1993; Fischman et al., 1994; Elezi et al., 1998; Bennett and O'Sullivan, 2001; Heckenkamp et al., 2002). This process of vessel re-narrowing is characterized by neointimal hyperplasia resulting in lumen loss and impaired vascular function. The vascular response to injury triggers a migratory and proliferative response within the smooth muscle cells resulting in intimal thickening (Noda-Heiny and Sobel, 1995; Libby and Tanaka, 1997; Elezi et al., 1998; Ward et al., 2000; Bennett and O'Sullivan, 2001; Heckenkamp et al., 2002; Lanza et al., 2002; Segev et al., 2002; Bhargava et al., 2003; Crook and Akyurek, 2003; Indolfi et al., 2003). In this regard, emphasis has been placed on developing pharmacological therapy aimed at reducing the proliferative response. Currently, two pharmacological agents have been approved for clinical use in the treatment of post-PTCA restenosis (Drachman et al., 2000; Heldman et al., 2001; Suzuki et al., 2001). Taxol and Rapamycin are being delivered through the use of coated coronary stents. Preliminary results suggest that these approaches offer significant protection against the restenosis process and validate the use of antiproliferative agents in the treatment of vascular proliferative disorders such as restenosis (Sousa et al., 2001; Gershlick et al., 2004; Stone et al., 2004).

As such, we have explored the use of RR inhibition as a new therapeutic target in ameliorating balloon mediated restenosis injury. The biochemical attributes of this enzyme make it amenable for the treatment of proliferative disorders since inhibition of RR blocks DNA synthesis and thus cell replication. Therefore, using as balloon model of arterial injury we have studied the effects of RR inhibition on the restenosis process.

Our results demonstrated that following balloon injury, one week of systemic administration of the RR inhibitors, Didox and Imidate, largely inhibited neointimal formation resulting in a 60% reduction in the intima/media ratio. Morphometric analysis revealed an  $\approx$  60% reduction in neointimal area with no significant change in the medial area between treated and untreated groups. However, there was a small but statistically significant decrease in medial area following Imidate dosing when this cohort was compared against HU and Didox. These results suggest that Imidate may have some negative effects on smooth muscle remodeling following medial injury that result in medial wall thinning.

These *in-vivo* studies demonstrate that inhibition of RR limits the extent of intimal hyperplasia following mechanical injury. However, these compounds possess a variety of chemical attributes which may contribute to their protective effects. Didox has been shown to inhibit NF $\kappa$ B and Tissue Factor while both compounds are potent free radical scavengers (Fritzer-Szekeres et al., 1997; Lee et al., 1997; Duilio et al., 2001; Shet et al., 2003; Turchan et al., 2003). Because of the myriad of effects elicited by these compounds, further experiments were carried out using the commercially available RR inhibitor, HU. Following the same dosing regimen for Didox and Imidate, HU afforded similar anti-restenotic efficacy, further supporting our observation regarding the importance of RR in the vascular response to injury.

Because the vascular response to injury is a chronic process, additional studies were performed in order to assess whether the protective effects elicited by early RR inhibition are maintained throughout the remodeling period. The RR inhibitors (Didox, Imidate and HU) were administered daily for one week and the extent of injury assessed at 6 weeks after balloon dilatation. This additional recovery time resulted in a >50% increase in the intima/media ratio demonstrating the progression of the lesion over time. Interestingly, Didox and HU treatment reduced the intima/media ratio by >70%, while slightly less efficacy was observed with Imidate. Moreover, the degree of protection afforded by these compounds was significantly increased when compared to the results of the two week study. This suggests that RR is an early target in the vascular response to injury and that inhibition of this enzyme affects the long term vascular remodeling associated with restenosis.

These results demonstrate that RR inhibition limits the degree of restenosis following arterial dilation injury. We believe these effects are mediated through an inhibition of SMC proliferation, as this process precedes neointimal formation. Therefore, studies were performed using a cell proliferation assay. Didox, Imidate and HU treatment resulted in arrest of cell division. Analysis of dNTP pools demonstrated a greater than 50% reduction in dATP levels further supporting that the observed effects of these drugs are mediated at least in part through the inhibition of ribonucleotide reductase. These effects were independent of any cytotoxic action these compounds may possess as flow cytometry revealed < 3% apoptotic cells following the dosing regimes tested. In addition, prior to dNTP analysis, cells were counted and viability assessed using trypan blue exclusion. Cell viability was greater than 95% among all groups.

As previously stated, Didox and Imidate possess various chemical attributes in addition to RR inhibition, many of which may confer protection against restenosis (Fritzer-Szekeres et al., 1997; Lee et al., 1997; Duilio et al., 2001; Shet et al., 2003; Turchan et al., 2003). Among these is their ability to scavenge free radicals, which may modulate SMC migration, a critical component of neointimal proliferation (Duilio et al., 2001; Turchan et al., 2003). Therefore, we tested the effects of Didox and Imidate on SMC proliferation using a wound scrape assay. Results demonstrated that both compounds almost completely inhibited SMC migration. This would suggest that inhibition of SMC proliferation may contribute to the protection afforded by these compounds. Similarly, a number of studies have demonstrated that HU, when oxidized, can release NO. (Stolze and Nohl, 1990; Pacelli et al., 1996). Because NO has been shown to inhibit cell migration we tested whether HU possesses anti-migratory properties which could be involved in its anti-restenotic effects (Sarkar et al., 1997; Sarkar and Webb, 1998). HU at concentrations up to 1 mM had no effect on SMC migration. This is an important observation and would suggest that inhibition of RR is the principal mechanism through which these compounds afford their protection as similar efficacy was seen with all compounds. However, if the in-vivo results are interpreted on a molar basis, HU (2.6 mmoles/kg/day) doses are 2-3 fold higher than that of Didox (1.2 mmoles/kg/day) and Imidate (0.9 mmoles/kg/day) and suggests that the effects of Didox and Imidate on SMC migration may contribute to the vascular protective effects observed with these two compounds.

Taken together, the in-vivo and in-vitro data demonstrate that activation of RR is a critical, early component in the proliferative response associated with vascular injury and that inhibition of this enzyme may reduce the vascular pathology associated with restenosis injury. Although the incidence of restenosis has markedly decreased with the advent of drug coated



stents, restenosis still occurs in up to 20 % of patients within the first year while results on late lumen loss are still being gathered (Drachman et al., 2000; Heldman et al., 2001; Grube et al., 2003; Kastrati et al., 2005; Kim et al., 2005). In addition, because the use of coated stents may increase the risk of thrombosis there is a need for agents which can be administered systemically in patients at high risk for thrombotic events (Morice, 2005). We believe that inhibition of RR may be a pathway that can be therapeutically targeted through either local or systemic delivery based on the low toxicity associated with current RR inhibitor therapy using HU. Additionally, our data suggests that RR is an early target in the restenosis process and as such, early pharmacological intervention may preclude chronic therapy and its associated adverse side effects. We believe that these observations have important therapeutic potential and implicate RR as a promising therapeutic target in the treatment of vascular proliferative disorders.

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## Footnotes

Authors contributed equally to this work. (J.C.H and M.M.B)

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### Legends for Figures

Figure 1. Effects of Didox, Imidate and HU on the histopathology associated with balloon dilatation injury. Examples are from carotid artery sections stained with hematoxylin and eosin. Panel (A) represents a control (uninjured) section of rat carotid artery. Panel (B) demonstrates the marked neointimal hyperplasia present at 2 weeks post balloon dilatation injury. Panel (C) demonstrates the inhibitory effects of Didox (200mg/kg/day) on neointimal proliferation in response to balloon injury. Panel (D) demonstrates the inhibitory effects of Imidate (200 mg/kg/day) on neointimal proliferation in response to balloon injury. Panel (E) demonstrates the inhibitory effects of HU (200 mg/kg/day) on neointimal proliferation in response to balloon injury. Note the marked reduction in neointimal (NI) thickness following Didox, Imidate and HU treatment.

Figure 2 A, B, C. Effects of Didox, Imidate and HU on histopathological changes following balloon injury of the rat carotid artery at 2 weeks post injury. Morphometric analysis was performed at the conclusion of the study. A.) Neointima formation. B.) Medial wall thickness. C.) Intima to media ratio. (Control) represents the uninjured contralateral artery. (Injured) represent the ipsilateral balloon dilated artery. (Didox) groups were administered the drug immediately following injury followed by daily administration for 6 days. (Imidate) groups were administered the drug immediately following injury followed by daily administration for 6 days. (HU) groups were administered the drug immediately following injury followed by daily administration for 6 days. The data represent the mean  $\pm$  SD. \* Significantly different at  $p < 0.05$  as compared to injured (untreated). ‡ Significantly different at  $p < 0.05$  among treated groups.

Figure 3 A, B, C. Effects of Didox, Imidate and HU on histopathological changes following balloon injury of the rat carotid artery at 6 weeks post injury. Morphometric analysis was performed at the conclusion of the study. A.) Neointima formation. B.) Medial wall thickness. C.) Intima to media ratio. (Control) represents the uninjured contralateral artery. (Injured) represent the ipsilateral balloon dilated artery. (Didox) groups were administered the drug immediately following injury followed by daily administration for 6 days. (Imidate) groups were administered the drug immediately following injury followed by daily administration for 6 days. (HU) groups were administered the drug immediately following injury followed by daily administration for 6 days. The data represent the mean  $\pm$  SD. \* Significantly different at  $p < 0.05$  as compared to injured (untreated). ‡ Significantly different at  $p < 0.05$  among treated groups.

Figure 4. Effects of Didox, Imidate and HU on SMC proliferation. Didox (0-200  $\mu$ M), Imidate (0-200  $\mu$ M) and HU (0-1000  $\mu$ M) were added to the SMC culture during the log phase of growth and incubated for 24 hours. Cells were then counted using a flow cytometer. Values represent the mean. (n = 4).

Figure 5. Effects of Didox, Imidate and HU on SMC migration. Didox (0-100  $\mu$ M), Imidate (0-100  $\mu$ M) and HU (0-1000  $\mu$ M) were added to the SMC culture in media containing 0.1 % serum and 10ng/mL PDGF. A wound scrape was then made and SMC migration was monitored for an additional 24 hours. The data represent the mean  $\pm$  SD. \* Significantly different at  $p < 0.05$  as compared to control.



Tables

Table 1. Effects of Didox, Imidate and HU on SMC dATP pools.

	Control	Didox ( $\mu\text{M}$ )					Imidate ( $\mu\text{M}$ )					Hydroxyurea ( $\mu\text{M}$ )			
<b>Dose</b>	NT	5	10	50	100	200	5	10	50	100	200	50	100	500	1000
<b>dATP levels</b> (pmoles/ $10^7$ cells)	303	297	260	203*	140*	126*	293	311	210	184*	173*	320	266	93	92
<b>S.D.</b>	50	35	26	20	40	31	31	17	47	41	39	30	42	11	17

Table 1. Effects of Didox, Imidate and HU on SMC dATP pools. Didox (0-200  $\mu\text{M}$ ), Imidate (0-200  $\mu\text{M}$ ) and HU (0-1000  $\mu\text{M}$ ) were added to the SMC culture during the log phase of growth and incubated for 24 hours. dNTP's were extracted and samples subjected to HPLC analysis. The data are presented as pmoles dATP/ $10^7$  cells and represent the mean  $\pm$  SD. \* Significantly different at  $p < 0.05$  as compared to control.

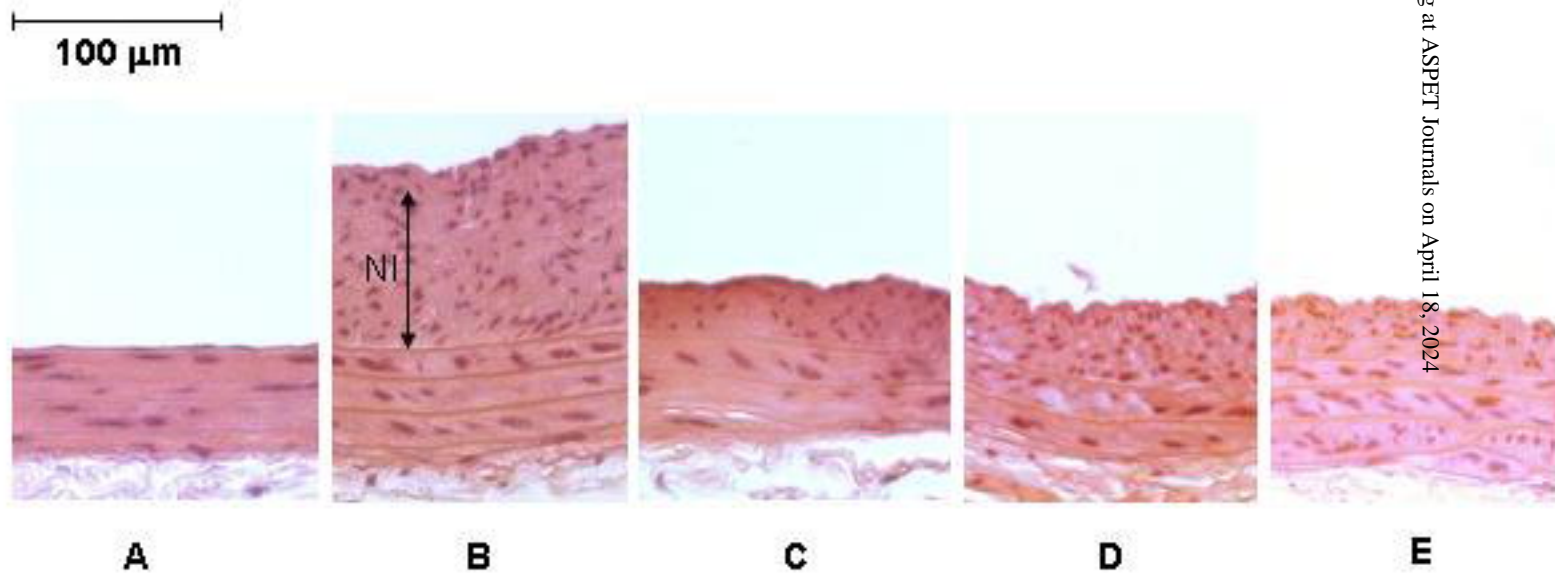


Fig. 1

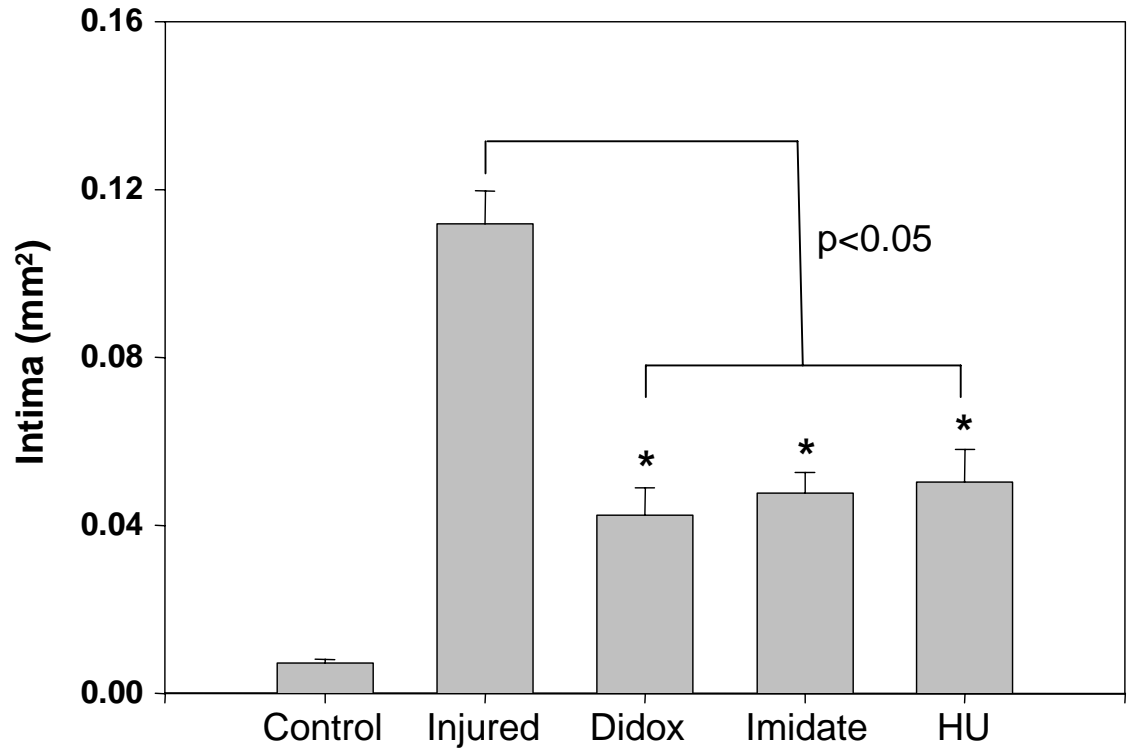


Fig. 2A

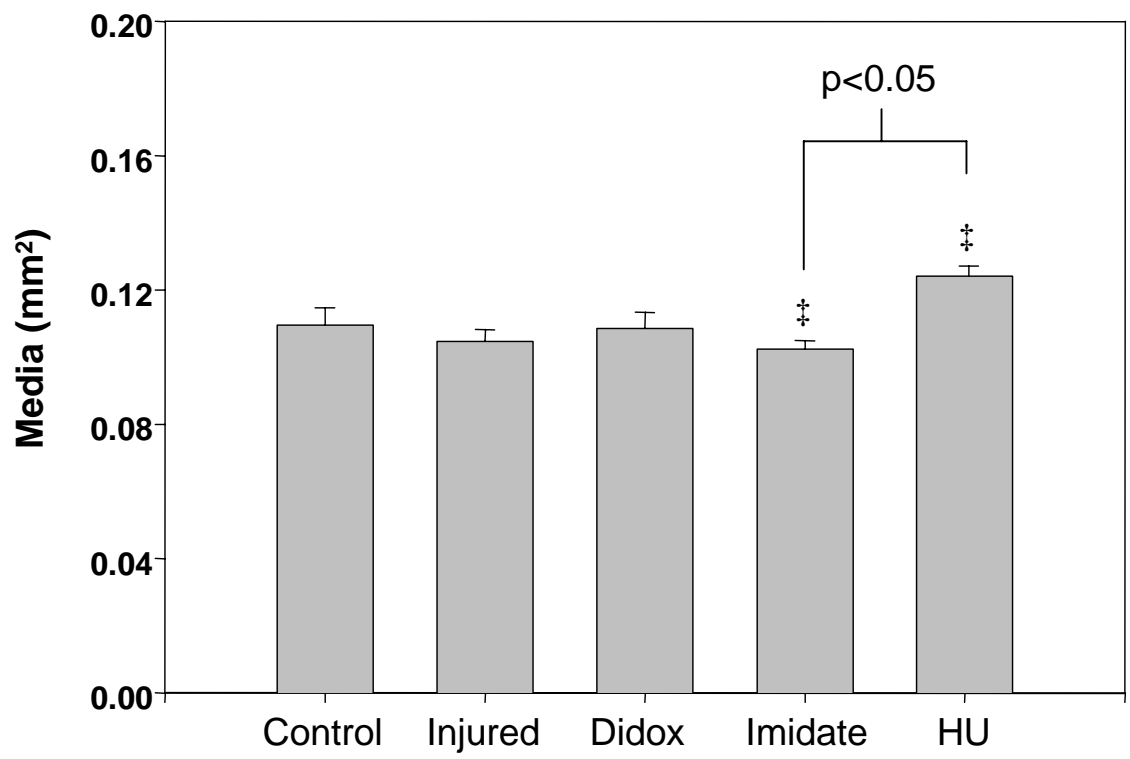


Fig. 2B

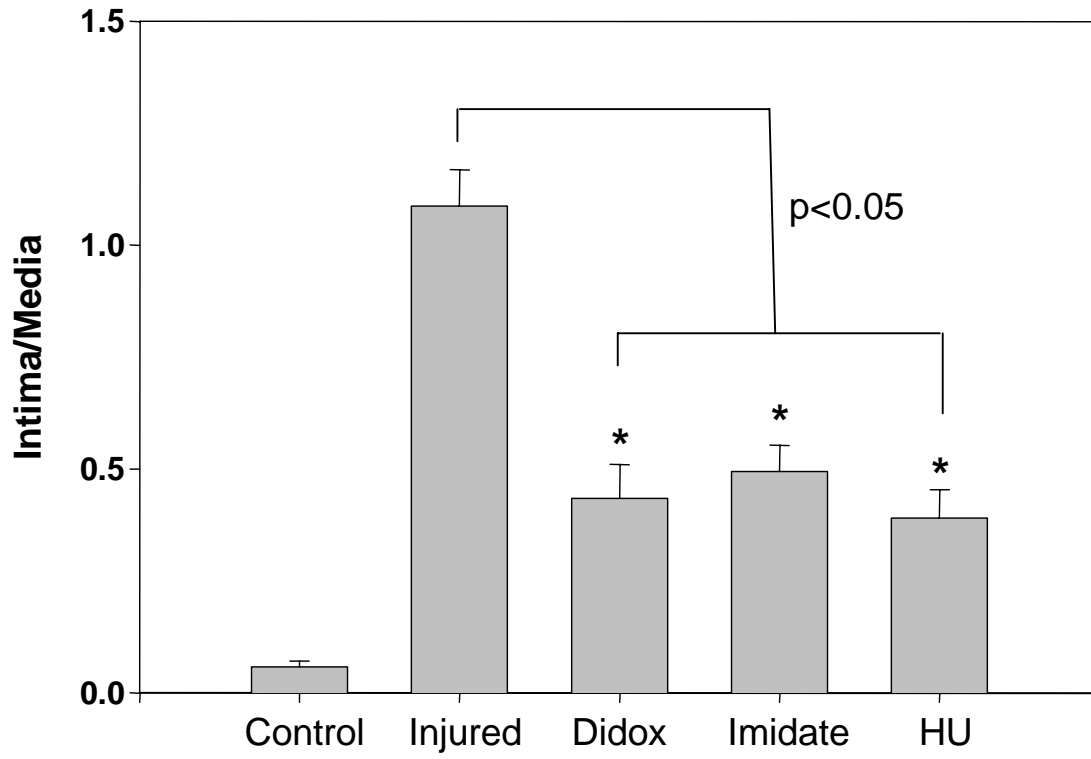


Fig. 2C

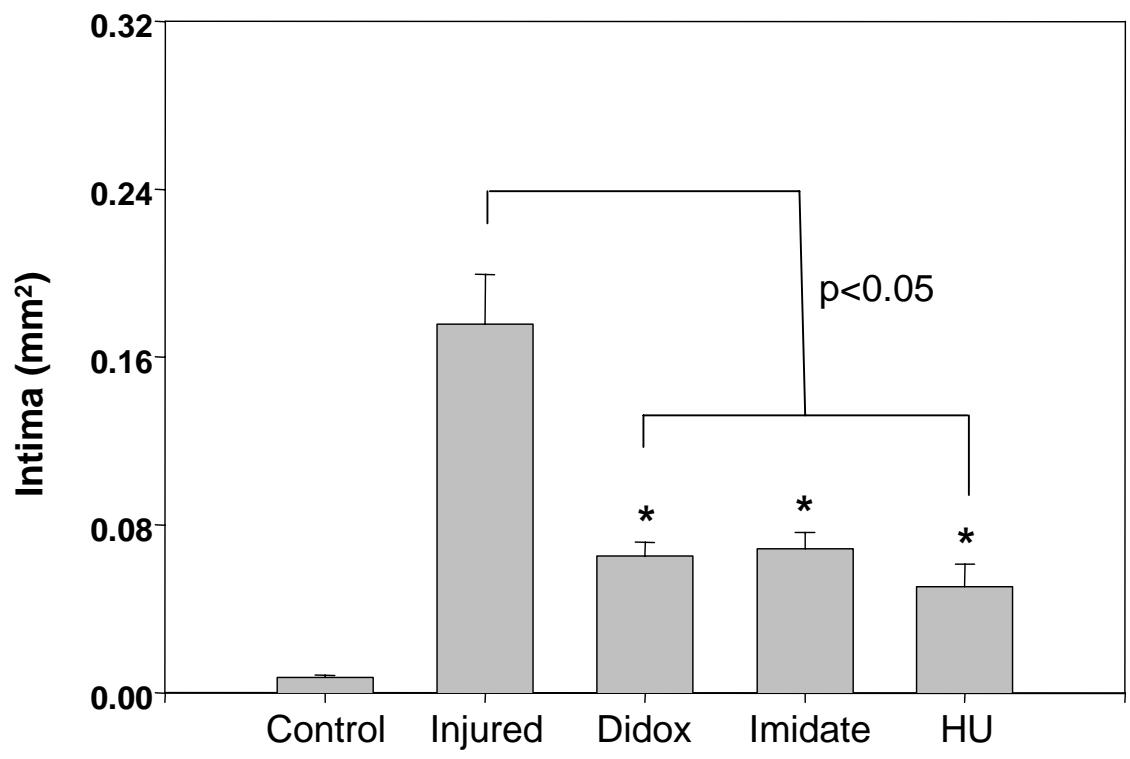


Fig. 3A

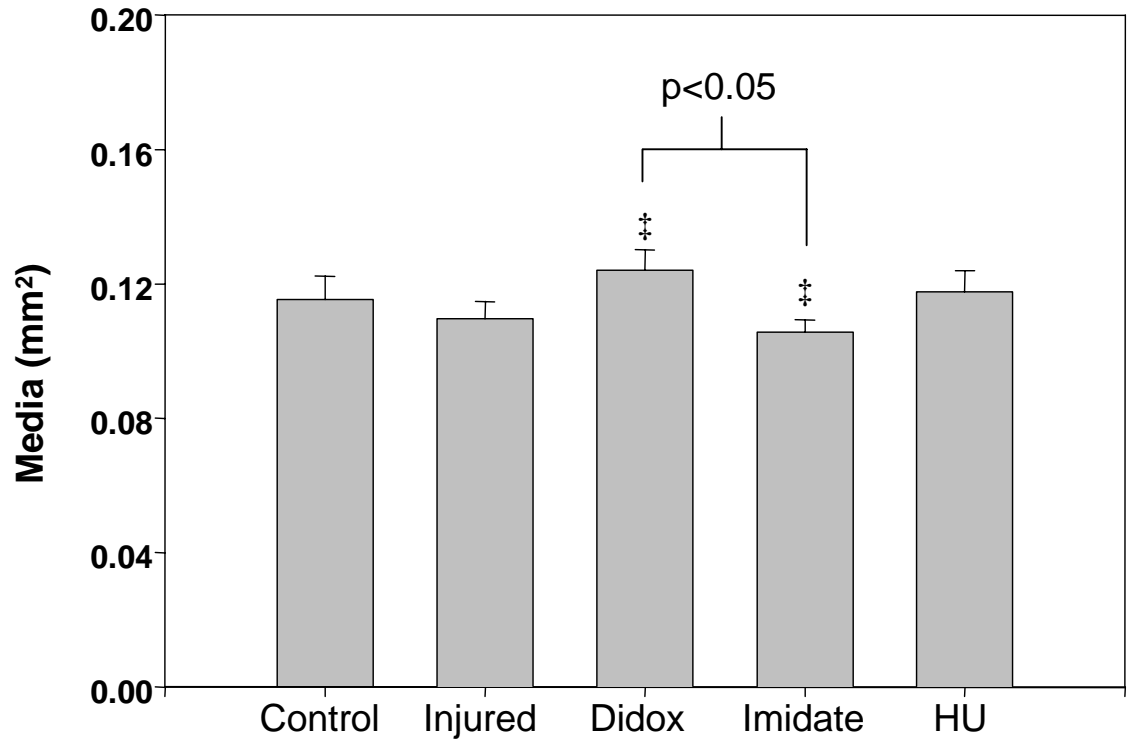


Fig. 3B

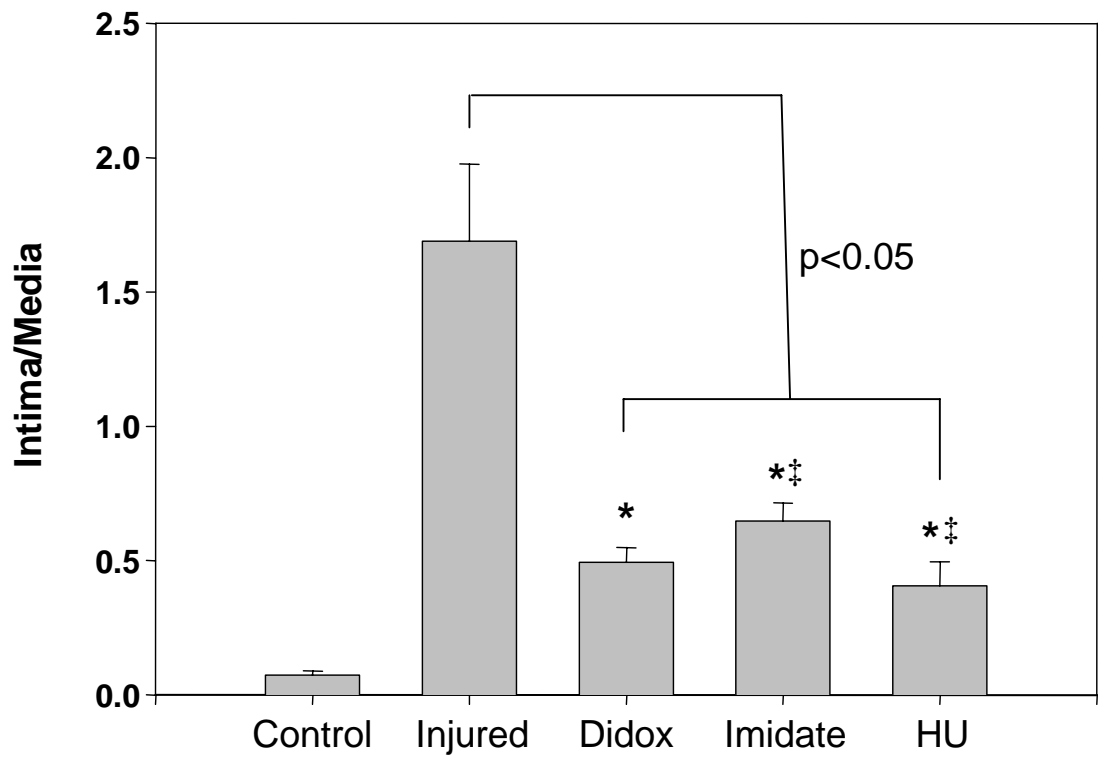


Fig. 3C



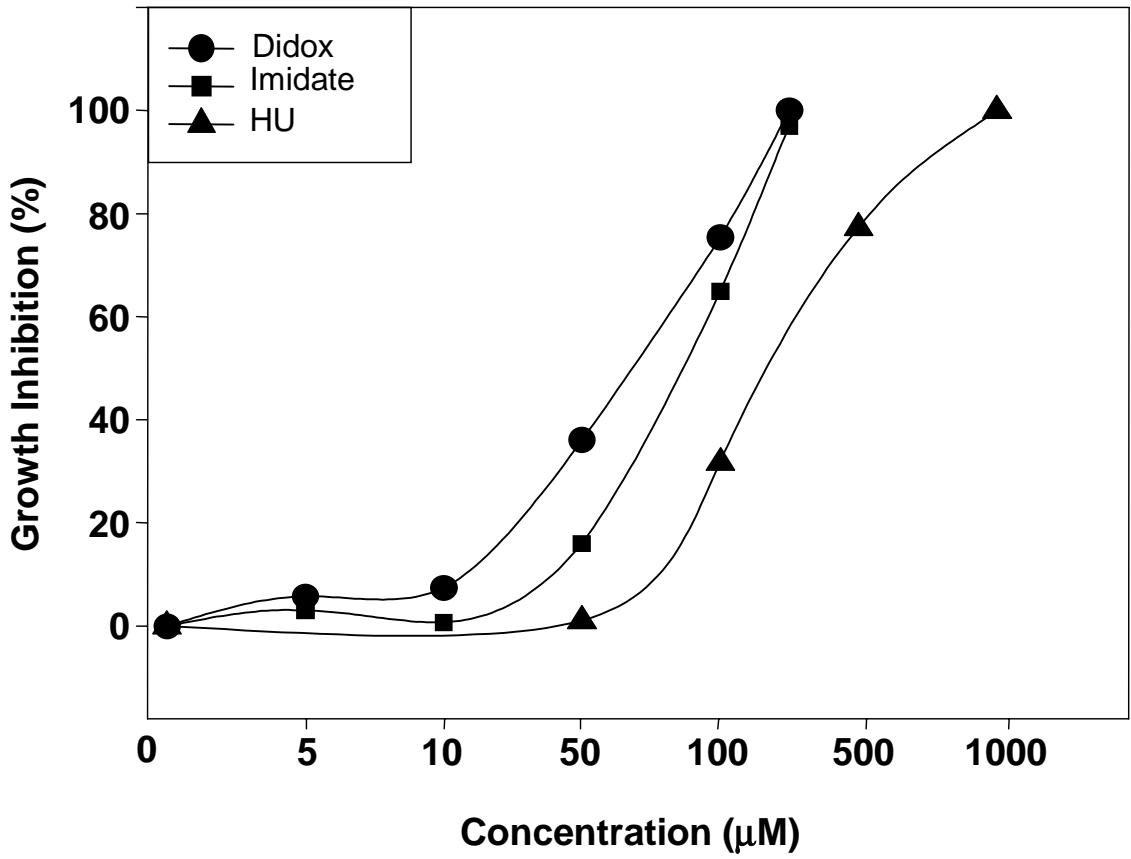


Fig. 4

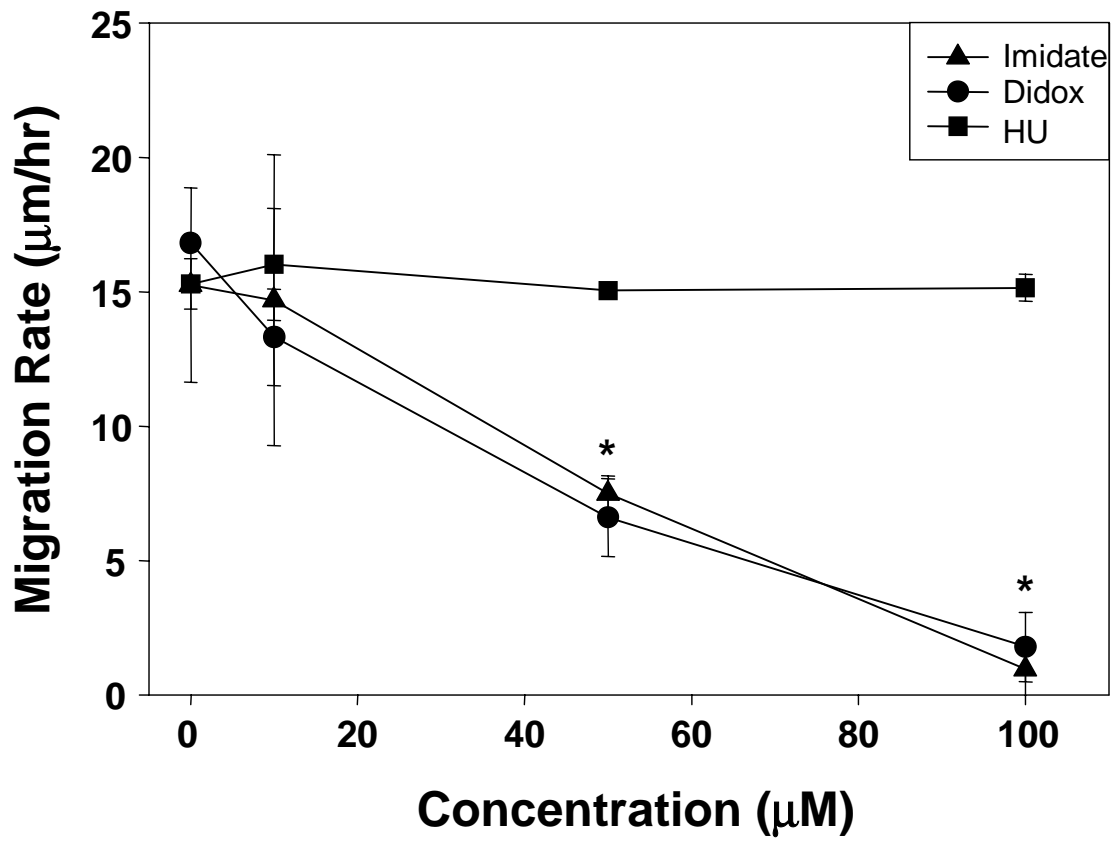


Fig. 5