Mitoxantrone suppresses vascular smooth muscle cell (VSMC) proliferation and balloon injury-induced neointima formation: An in vitro and in vivo study

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ABSTRACT

Neointima formation, which occurs after vascular injury due to vascular disease or interventions such as angioplasty and stent placement, is a complex process that involves multiple molecular and cellular mechanisms. The inhibition of neointima formation is vital to prevent restenosis of blood vessels. In the present study, we investigated whether the systemic administration of mitoxantrone can inhibit neointima formation, and evaluated the underlying mechanisms under in vitro and in vivo experimental conditions. In vitro, rat and human vascular smooth muscle cells (RVSMCs and HVSMCs) were stimulated with platelet-derived growth factor-BB (PDGF-BB) and treated with mitoxantrone or DMSO as a control. In vivo, 54 male Sprague-Dawley rats were subjected to carotid artery balloon injury and then intravenously administered with mitoxantrone. Cell proliferation was determined using the CCK-8 assay. Cell cycle analysis was performed using flow cytometry, and protein expression was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. We used monoclonal mouse anti-bromodeoxyuridine (BrdU) antibody for the detection of BrdU and anti-Type II topoisomerase antibody for staining Type II topoisomerase (Topo II), one week after the balloon injury. In both RVSMCs and HVSMCs, mitoxantrone treatment induced Topo II degradation, as well as suppressed DNA replication, cell cycle progression, and VSMC proliferation. A reduction in intimal hyperplasia, intimal-to-medial area ratio, and Topo II level was observed in mitoxantrone-treated rats, as compared to the control (saline) group. Overall, our results indicate that systemic administration of mitoxantrone can reduce neointimal hyperplasia and, thus, represents a suitable option for restenosis treatment.

KEY WORDS: Neointima formation; mitoxantrone; vascular smooth muscle cells; VSMCs; Type II topoisomerase; balloon injury model; proliferation

INTRODUCTION

Stent implantation and balloon angioplasty are standard surgical procedures for atherosclerotic vascular diseases, such as coronary artery disease and arteriosclerosis obliterans of the lower extremities. The prevalence of these conditions increases with age. It is well established that the proliferation and migration of vascular smooth muscle cells (VSMCs) play a major role in the development of restenosis after the treatment [1,2]. Currently, there is an urgent need for novel strategies for the pharmacologic inhibition of the proliferation and migration of VSMCs.

Major advances have been made in the development of drug-eluting stents and balloons over the past two decades, including the development of first-generation to fourth-generation devices and the use of antiproliferative drugs, from sirolimus and paclitaxel to everolimus and biolimus [3]. Although new generation stents have improved features, they are still associated with high restenosis rates and late stent thrombosis following surgery. Moreover, due to a high operative risk, up to 30% of patients are not candidates for such interventions [4]. Hence, it is important to understand the complex mechanisms underlying restenosis and develop techniques to reduce neointimal hyperplasia.

Increasing evidence has shown that abnormal VSMC proliferation and migration commonly occur during the
pathogenesis of atherosclerosis and restenosis after angioplasty [1]. In particular, platelet-derived growth factor (PDGF) can induce the proliferation, migration, and angiogenesis of VSMCs [5-7]. Moreover, the restenotic process may be initiated by a vascular injury caused during balloon angioplasty, which further stimulates VSMCs to proliferate and migrate from the basal and medial layers to the intimal layer of the vessel wall, eventually leading to uncontrolled neointimal hyperplasia.

The cell cycle consists of G0, S, G1, and M phases and is promoted by various mitogenic stimuli during cell proliferation. The regulation of cell cycle gene expression plays an important role in controlling cell proliferation. Following vascular injury, mitogens stimulate VSMCs to proliferate; namely, the cells enter the cell cycle from the G0 phase [1,8]. Different pharmacological agents, including antiproliferative, antithrombotic, and antiplatelet agents, can inhibit cell cycle events, and thus suppress cell proliferation [9].

Mitoxantrone is a synthetic antineoplastic drug with numerous activities demonstrated in various animal cancer models; moreover, it has been used in the clinical treatment of different types of human cancers, particularly prostate cancer, breast cancer, and gastrointestinal stromal tumor, as well as lymphomas and leukemia [10-13]. The inhibition of Type II topoisomerase (Topo II) activity, by targeting the enzyme-DNA complex, is the main anti-tumor mechanism of mitoxantrone [14]. Moreover, other pharmacological activities of mitoxantrone have been indicated, including strong DNA binding via intercalation or electrostatic interactions [15,16], tubulin binding and possible inhibition of microtubule assembly [17], inhibition of several kinases and GTPases [18-20], and induction of calcium release [16].

A preliminary study reported that stent grafts loaded with mitoxantrone inhibited the development of neointimal hyperplasia after a vessel injury [21]. However, the mechanism of action was not investigated, and the regenerative processes were also prevented, possibly due to a local overdose. Furthermore, it was unclear whether the systemic administration of mitoxantrone could lead to better outcomes by inhibiting neointima formation. In this study, we aimed to determine whether systemic administration of mitoxantrone can inhibit balloon injury-induced neointima formation by suppressing VSMC proliferation. Moreover, we sought to investigate the mechanisms underlying this process. Our findings could serve as the basis for a new therapeutic method for proliferative vascular diseases, such as restenosis after surgery.

MATERIALS AND METHODS

Reagents

Bromodeoxyuridine (BrdU), BrdU labeling mix, mouse anti-BrdU antibody, FITC-conjugated goat anti-mouse antibodies, and mitoxantrone were purchased from Sigma (St. Louis, MO, USA). PDGF-BB and Anti-Topoisomerase II beta antibody were purchased from Santa Cruz (Santa Cruz, CA, USA). Topoisomerase II antibody was purchased from Novus (Littleton, CO, USA). Anti-beta actin antibody was purchased from Roche (Pleasanton, CA, USA). Mitoxantrone was dissolved in dimethyl sulfoxide (DMSO) and further diluted in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) for in vitro cell culture experiments.

Ethics statement

All rats used in this study were purchased from Guangdong Medical Laboratory Animal Center. All experimental procedures were authorized by the Research Ethics Committee of The First Affiliated Hospital, Sun Yat-Sen University.

Primary human VSMCs (HVSMCs) were obtained from the femoral artery of a healthy organ donor with the donor’s consent and approval of the Research Ethics Committee of The First Affiliated Hospital of Sun Yat-Sen University.

Primary cell culture

Rat VSMCs (RVSMCs) were isolated from Sprague-Dawley rats and HVSMCs were obtained from the femoral artery of a healthy organ donor. The cell culture methods used for the primary RVSMCs and HVSMCs have been described previously [22,23]. The cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified incubator containing 5% CO2. The VSMC purity was confirmed by immunocytochemistry with an alpha-smooth muscle actin monoclonal antibody. The fourth to eighth passages of the VSMCs were used in this study.

Cell proliferation assay

Cell proliferation was determined using the CCK-8 assay. In brief, the primary VSMCs were seeded in 96-well plates (~20% confluent cells) with growth medium containing 20 μg/L PDGF-BB as an attractant, and were incubated for 12 hours. DMSO (as the control) or different amounts of mitoxantrone dissolved in the culture medium were added into the wells, and the cells were further incubated for 48 hours. Thereafter, the medium was removed, 10 μl of CCK-8-containing medium was added, and the cells were incubated at 37°C for 1 hour. The absorbance was measured at 450 nm. The relative cell viability was calculated as the ratio of the number of mitoxantrone-treated cells versus the number of DMSO-treated cells.

Cell cycle analysis

The distribution of cell cycle stages was determined using flow cytometry. In brief, PDGF-BB-stimulated cells treated with
mitoxantrone or DMSO were collected and washed twice with PBS. The cells were then fixed in 70% ethanol for at least 1 hour and stained with propidium iodide buffer (50 g/mL RNase, 0.1% Triton X-100, 0.1 mM EDTA, and 50 μg/mL propidium iodide) for 0.5 hour before analysis. The samples were analyzed using a FACS machine (Becton, Dickinson and Company, USA).

**Immunoblotting analysis**

PDGF-BB-stimulated VSMCs treated with mitoxantrone or DMSO were collected and lysed by directly adding 1× sodium dodecyl sulfate (SDS) sample buffer into the 12-well plates. The VSMC proteins were resolved by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto nitrocellulose membranes. The blots were successively probed with primary antibody and horseradish peroxidase (HRP)-conjugated secondary antibody. The results were detected by an BeyoECL Plus detection kit (Beyotime, China), and the X-ray films were developed in a film developer.

**Balloon injury model in a rat carotid artery**

A total of 44 male Sprague-Dawley rats, weighing 300-350 g, were classified into three groups: a saline control group and two mitoxantrone-treated groups (n = 18 each).

All rats were anesthetized with chloral hydrate (35 mg/kg intraperitoneal [IP] injection) and subjected to balloon injury of the right common carotid artery, as reported previously [24]. Each rat received caudal vein injection of 0.2 or 1.0 mg/kg of mitoxantrone hydrochloride dissolved in 2 ml of normal saline, twice weekly, from the first day after the surgery until sacrifice. Each rat in the control group was injected with 2 ml of normal saline.

Six rats in each group were used for immunohistochemistry staining, to determine the Topo II and BrdU levels 7 days after the surgery; in the remaining 12 rats in each group neointima formation was measured in the artery, 28 days following the surgery. The rats were sacrificed by pressure fixation of the right common carotid artery.

**Morphometric analysis and immunohistochemistry**

Carotid arteries were isolated from the euthanized rats and were perfusion-fixed in situ with 4% formaldehyde. Three circular cross-sections (6 μm in thickness) were cut from the middle part of the artery and stained with hematoxylin-eosin (H&E). The intimal hyperplasia (IH) area and the intima-media ratio (IMR) were measured and calculated, as reported previously [25]. The IH and IMR were analyzed using Image-Pro Plus 6.0 software.

Six rats per group received an IP injection of 100 mg/kg of BrdU labeling mix 3 days after the surgery and were sacrificed for immunohistochemical analysis 7 days after the surgery, as reported previously [26]. The BrdU-incorporated cells in the carotid arteries were detected using a mouse monoclonal anti-BrdU antibody. The number of positive cells within the intimal lesions was counted based on the red fluorescent protein (RFP) signals. Three sections from each animal were analyzed. Topo II staining was performed using an anti-Topoisomerase II antibody. The images were analyzed by calculating the integrated optical density (IOD) value.

**Evaluation of rat’s general health condition**

Serum samples were collected from the inferior vena cava at the time of sacrifice, to determine the blood cell count, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) levels. Liver, kidney, and lung, tissues were collected for histomorphological analysis. All rats were weighed weekly from the time of the operation until sacrifice.

**Statistical analysis**

Data are presented as mean ± standard deviation (SD). For statistical analysis, we performed one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls post-test. All statistical analyses were performed using Statistical Product and Service Solutions 22.0 software (SPSS 22.0) [IBM Corp, Armonk, NY]. A value of p < 0.05 was indicative of statistical significance.

**RESULTS**

Mitoxantrone induces Topo II degradation and inhibits VSMC proliferation in vitro

To investigate the effects of mitoxantrone on VSMC proliferation, we examined the viability of mitoxantrone-treated VSMCs (Figure 1A). The human and rat VSMCs were incubated with various concentrations of mitoxantrone for 48 hours. The cell viability was then measured using the CCK-8 assay. Mitoxantrone showed significant inhibitory effects on the proliferation of both PDGF-BB-stimulated human and rat VSMCs, and the inhibitory activities were dose-dependent. The inhibitory concentration 50% (IC$_{50}$) values of mitoxantrone for human and rat VSMCs were 0.35 and 0.67 μg/mL, respectively.

As the inhibition of cell proliferation by blocking the Topo II-DNA complex is assumed to be the anti-tumor mechanism of mitoxantrone [14], we analyzed the expression of Topo II in mitoxantrone-treated VSMCs by immunoblotting. The PDGF-BB-stimulated human and rat VSMCs treated with mitoxantrone were collected for immunoblotting; the cells treated with DMSO and untreated cells were used as negative
controls. The results indicated that mitoxantrone induced total degradation of Topo II in both human and rat VSMCs (Figure 1B). Thus, we suggest that mitoxantrone inhibits human and rat VSMC proliferation by blocking the Topo II activity, which is a vital factor in several cellular processes such as DNA replication, transcription, and chromosome condensation.

Effects of mitoxantrone on cell cycle progression

The cell cycle is a series of events, including cell growth, DNA replication, chromosome separation, and separation of all cell components. To determine the effects of mitoxantrone on cell cycle progression, we measured cellular DNA content by flow cytometry. The RVSMCs treated with mitoxantrone showed an increase in the S-phase population and a decrease in the G1-population in a dose-dependent manner, as compared to the negative DMSO control group (Figure 2A and B). In particular, the percentage of cells in the S phase increased from 8.3% in the DMSO control group to 14.8%, 19.7%, and 24.2% in the cells treated with 0.25, 0.5, and 1.0 µg/mL mitoxantrone, respectively. Similar results were also observed for the HVSMCs (Figure S1A and B).

The increased S-phase population most likely involved cells with incomplete DNA replication, and this hypothesis was also supported by the results of the BrdU incorporation assay (Figure 2C and D). The BrdU incorporation assay labels cells undergoing DNA replication, which is an indicator of cell proliferation. The PDGF-BB-stimulated human and rat VSMCs were treated with mitoxantrone for 24 hours and then pulse-labeled with BrdU for 1 hour; the BrdU-positive cells were then visualized by immunostaining. The results showed that, compared to the DMSO control cells, mitoxantrone markedly reduced the number of BrdU-positive RVSMCs (Figure 2C and D) and HVSMCs (Figure S1C and D), and completely inhibited DNA replication at higher concentrations (Figure 2C and D; Figure S1C and D), which is consistent with the essential role of Topo II in DNA replication. These results, along with the data from flow cytometry (Figure 2A and B; Figure S1A and B), strongly suggest that mitoxantrone can induce Topo II degradation and inhibit DNA replication and proliferation of human and rat VSMCs.

Mitoxantrone inhibits neointimal hyperplasia induced by balloon injury

To determine the effects of mitoxantrone on neointima formation in vivo, we subjected the rats to balloon injury of the right common carotid artery and intravenously administered mitoxantrone to these rats. The rats in the control group were also subjected to the balloon injury but treated with saline. The balloon injury resulted in a loss of endothelium and the development of IH, as examined on day 28 after the balloon injury (Figure 3A). According to the computer-assisted morphometric analysis, the mean IH area in the control saline group was 0.22 mm², whereas the IH area was markedly reduced to 0.073 and 0.046 mm² in the 0.2 and 1.0 mg/kg mitoxantrone-treated animals, respectively (Figure 3B). Furthermore, both the IMR and IH were lower in the 1.0 mg/kg mitoxantrone-treated than in the 0.2 mg/kg mitoxantrone-treated group (Figure 3B and C), suggesting a protective role of mitoxantrone in inhibiting IH. In addition, the wound from the balloon injury healed well in the mitoxantrone-treated rats (Figure 3A).

Topo II expression is negatively correlated with mitoxantrone dosage in neointima formation

The immunohistochemical staining of sections from the balloon-injured arteries on day 7 revealed that Topo II was significantly inhibited in the mitoxantrone-treated groups in a dose-dependent manner, as compared to the control group (Figure 3D and E). These data suggest that mitoxantrone inhibited Topo II expression during the neointima formation.
Mitoxantrone inhibits cell proliferation in vivo

The BrdU labeling of the carotid arteries harvested on day 7 following the balloon injury and mitoxantrone treatment showed a significant decrease in the number of BrdU-positive cells in a dose-dependent manner, as compared to the control saline group (Figure 4), indicating that mitoxantrone inhibited the cell proliferation in vivo.

Evaluation of the rat’s general health condition

The serum AST, ALT, and LDH levels were in the normal range in both the mitoxantrone-treated and control groups (Table S1). Moreover, no significant pathological changes were observed in the liver, kidney, or lung tissues in all animals (Figure S2A). The animals showed a steady increase in the body weight during the course of the experiment, although the increase was somewhat slower in the mitoxantrone-treated rats (Figure S2B), which is consistent with the inhibitory effects of mitoxantrone on cell proliferation. These results indicate that there were no obvious side effects in the rats that were systematically administered with up to 1 mg/kg of mitoxantrone.

DISCUSSION

High rates of restenosis remain a major problem in patients undergoing balloon angioplasty and other procedures that cause vascular damage. At present, drugs are commonly used to inhibit the formation of neointimal hyperplasia after surgery. Furthermore, animal model and clinical studies have demonstrated that antiproliferative drug-coated coronary stents could inhibit neointima formation [27–29]; for instance, both taxol- and sirolimus-eluting coronary stents have been approved for clinical use by the U.S. Food and Drug Administration (FDA). Mitoxantrone has been used as a cytostatic drug for treating prostate cancer and gastrointestinal stromal tumors [30,31]. Moreover, mitoxantrone displays immunosuppressive activity and has been approved by the FDA for the treatment of multiple sclerosis [32].

A preliminary study suggests that the use of mitoxantrone-eluting stents can reduce neointimal hyperplasia [21];...
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However, in that study, the regenerative processes were prevented and the underlying mechanisms could not be clarified. Moreover, it was unclear whether the systemic administration of mitoxantrone could lead to better outcomes by inhibiting neointima formation. In this study, we found that mitoxantrone had a strong antiproliferative effect on VSMCs in vitro and significantly inhibited the neointima formation in vivo. Moreover, we observed that the low dose (0.2 mg/kg) of mitoxantrone suppressed the neointimal formation by 62%, and the higher dose (1 mg/kg) of mitoxantrone inhibited the neointimal formation by approximately 80%, which is significantly higher compared to the suppressive effect of other drugs, such as taxol (70%)[26]. Furthermore, the IMR in mitoxantrone-treated rats was also markedly reduced, namely, it was 1.49 ± 0.22, 0.64 ± 0.08, and 0.44 ± 0.10 in the vehicle control, low-dose, and high-dose mitoxantrone groups, respectively. In previous animal model studies, the IMRs of the vehicle and sirolimus-treated groups were 1.4 ± 0.2 and 0.75 ± 0.1, respectively [33]. Thus, even a low dose of mitoxantrone showed significant inhibitory effects on neointima formation. Our results indicate that mitoxantrone is significantly more efficient in inhibiting neointimal hyperplasia, as compared to new generation drugs such as everolimus and biolimus [34-36]. Moreover, the mitoxantrone administration did not result in

FIGURE 3. Mitoxantrone inhibits neointimal hyperplasia in a dose-dependent manner after arterial injury. (A) Representative hematoxylin and eosin-stained carotid artery sections on day 28 after the injury in each group (n = 12). (B) Quantification of the intimal hyperplasia (IH) area. (C) The ratio of intimal area to the medial area of the injured rat carotid arteries was determined. (D) Topoisomerase II (Topo II) expression in the intima of the injured arteries on day 7 after the surgery (n = 6 for each group), as determined by immunohistochemical staining. I, M, and A represent intima, media, and adventitia, respectively. The space between the two arrows represents the intima area. The brown and black signals indicate Topo II staining. (E) Integrated optical density (IOD) of the Topo II signal in the intima area of the artery (*p < 0.05, **p < 0.01).
any significant pathological changes of the general condition and vital organs in the treated animals.

We further found that the antiproliferative activity of mitoxantrone was associated with DNA replication defects and cell cycle arrest in the S phase of VSMCs. A previous study indicated that pharmacological interventions to regulate the cell cycle could inhibit VSMC proliferation and block neointimal hyperplasia [37]. In this study, we found that mitoxantrone could induce Topo II degradation and inhibit DNA replication and cell proliferation in VSMCs, both in vitro and in vivo.

As topoisomerases play crucial roles in DNA replication and cell proliferation, their inhibition can suppress cancer cell proliferation; in fact, this is the mechanism of action of several anticancer drugs, including etoposide, doxorubicin, and camptothecin. These drugs induce DNA strand breaks by forming a complex with DNA and topoisomerase, and thus inhibit the topoisomerase activity, leading to DNA damage, cell cycle arrest, and apoptosis. In this study, we evaluated the effects of mitoxantrone using human and rat VSMCs as in vitro models. Our results showed that mitoxantrone could induce the total degradation of Topo II in human and rat VSMCs and inhibit DNA replication and cell proliferation in vitro. Our study also showed that mitoxantrone reduced the Topo II levels, suppressed VSMC proliferation, and decreased the IH and IMR in the carotid arteries in vivo. Nevertheless, other mechanisms may also be involved in the mitoxantrone inhibition of VSMC proliferation, and this should be further investigated.

CONCLUSION

In summary, this study provides new insights into the molecular mechanisms underlying the inhibition of VSMC proliferation by mitoxantrone. Our results indicate that mitoxantrone can inhibit VSMC proliferation in vitro and in vivo as well as suppress balloon injury-induced neointima formation in vivo. Thus, mitoxantrone could be effective in reducing restenosis rates after angioplasty.

ACKNOWLEDGMENTS

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

REFERENCES

Mitoxantrone prevents restenosis following vascular injury

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SUPPLEMENTAL DATA

**FIGURE S1.** Mitoxantrone induced cell cycle arrest in the S phase and inhibited human vascular smooth muscle cell (HVSMC) proliferation. (A) Effects of mitoxantrone on the cell cycle. The distribution of cell cycle stages in the HVSMCs treated with different concentrations of mitoxantrone was determined by flow cytometry. Data from 1 of 3 independent experiments are shown. (B) The average percentages of cells in different cell cycle phases were determined from the 3 separate experiments. (C) Quantification of the bromodeoxyuridine (BrdU)-positive cells from the 3 experiments. (D) The mitoxantrone-treated HVSMCs were pulse-labeled with BrdU. The BrdU-positive cells were visualized by immunostaining with an anti-BrdU antibody (*p < 0.05, **p < 0.01 versus dimethyl sulfoxide [DMSO] group).
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Results are expressed as mean±standard deviation, n=12. The drug dosage is represented as mg/kg. RBC: Red blood cells; WBC: White blood cells; HGB: Hemoglobin; PLT: Platelets; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; and LDH: Lactate dehydrogenase. The main blood and biochemical indicators of the three groups of rats were within the normal range.