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Inhibition of In-Stent Stenosis by Oral Administration of Bindarit in Porcine Coronary Arteries

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Objective—We have previously demonstrated that bindarit, a selective inhibitor of monocyte chemotactic proteins (MCPs), is effective in reducing neointimal formation in rodent models of vascular injury by reducing smooth muscle cell proliferation and migration and neointimal macrophage content, effects associated with the inhibition of MCP-1/CCL2 production. The aim of the current study was to evaluate the efficacy of bindarit on in-stent stenosis in the preclinical porcine coronary stent model.

Methods and Results—One or 2 bare metal stents (Multi-Link Vision, 3.5 mm) were deployed (1:1.2 oversize ratio) in the coronary arteries of 42 pigs (20 bindarit versus 22 controls). Bindarit (50 mg/kg per day) was administered orally from 2 days before stenting until the time of euthanasia at 7 and 28 days. Bindarit caused a significant reduction in neointimal area (39.4%, \(P<0.001\), n = 9 group), neointimal thickness (51%, \(P<0.001\)), stenosis area (37%, \(P<0.001\)), and inflammatory score (40%, \(P<0.001\)) compared with control animals, whereas there was no significant difference in the injury score between the 2 groups. Moreover, treatment with bindarit significantly reduced the number of proliferating cells (by 45%, \(P<0.05\); n = 6 group) and monocyte/macrophage content (by 55%, \(P<0.01\); n = 5–6 group) in stented arteries at day 7 and 28, respectively. These effects were associated with a significant (\(P<0.05\)) reduction of MCP-1 plasma levels at day 28. In vitro data showed that bindarit (10–300 \(\mu\)mol/L) reduced tumor necrosis factor-\(\alpha\) (50 ng/mL)–induced pig coronary artery smooth muscle cell proliferation and inhibited MCP-1 production.

Conclusion—Our results show the efficacy of bindarit in the prevention of porcine in-stent stenosis and support further investigation for clinical application of this compound. (Arterioscler Thromb Vasc Biol. 2011;31:2448-2454.)

Key Words: pharmacology  ■ restenosis  ■ stent  ■ bindarit
duction. However, there are many instances where rodent models of neointimal formation have lacked efficacy in predicting the success of interventions to inhibit restenosis in humans. Therefore, the aim of the present study was to evaluate the efficacy of bindarit on in-stent stenosis in the preclinical porcine coronary stent model.

Methods

Animals

Male large-white/Landrace intact pigs (20–24 kg, 10 weeks old, SAC Commercial Ltd, Edinburgh, United Kingdom) on a 12-hour light/dark cycle, with free access to water and twice-daily food, were maintained at the Biological Procedures Unit, University of Strathclyde. All procedures were performed in accordance with local ethical and UK Home Office regulations.

Bindarit Administration

Bindarit (2-methyl-2-[(1-phenylmethyl)-1H-indazol-3-yl][methoxy] propanoic acid, MW 324.38) was synthesized by Angelini (Angelini Research Center, Azienda Chimica Rumerie Angelini Francesco, Rome, Italy). Pigs were dosed with bindarit (50 mg/kg per day) in 2 divided doses administered morning and evening (12-hour interval). Dosing started 2 days before stenting and continued daily for the 7 or 28 days after stent implantation. The bindarit powder was mixed with yogurt and squirted into the pigs’ mouths. Control animals received vehicle alone (yogurt containing no bindarit).

The dose regimen of bindarit was chosen based on earlier results from a swine renovascular hypertension model and pharmacokinetic studies in male Gottingen minipigs (10–12 kg, Harlan) showing that bindarit is well absorbed when administered by the oral route.

Porcine Coronary Stent Model

Pigs were premedicated with aspirin (150 mg oral, Teva, Leeds, United Kingdom) and clopidogrel (150 mg oral, Sanofi-Aventis, Guildford, United Kingdom) over a 24-hour period before surgery. Pigs were sedated by an injection of tiletamine/zolazepam (100 mg Zoletil IV, Virbac, Suffolk, United Kingdom) and propofol (30 mg Propofol IV, Abbott Laboratories Ltd, Maidenhead, UK) in oxygen/nitrous oxide. Unfractionated heparin (100 U/kg IV, Leo Laboratories Ltd, Maidenhead, UK) was given at the start of the procedure. Antimicrobial cover in the form of cefuroxime and ampicillin (350 mg of Ampicilin LA IM, Intervet, Welwyn Garden City, United Kingdom) for antibiotic cover, immediately after the procedure. Animals were recovered and received a normal diet, with supplementation of 75 mg of aspirin orally every 2 days and 75 mg of clotidogrel orally every 2 days for the duration of the study.

Injury and Inflammatory Score

The injury score was calculated as previously reported by Gunn et al. Briefly, we considered both deep injury and stretch as follows: 0=no impression of metal on media; 1=deformation of the IEL by <45°; 2=deformation of the IEL by 45–90°; 3=rupture of the IEL; 4=rupture of the external elastic lamina (that is complete medial rupture).

The inflammatory score was calculated as previously reported by Kornowski et al. Briefly, we considered the extent and density of the inflammatory infiltrate in each individual strut, and the grading used was as follows: 0=no inflammatory cells surrounding the strut; 1=light, circunferential lymphohistocytic infiltrate surrounding the strut; 2=localized, moderate to dense cellular aggregate surrounding the strut noncircumferentially; 3=circumferential dense lymphohistocytic cell infiltration of the strut. The injury and inflammatory score for each cross section were calculated as the sum of the individual injury or inflammatory scores, divided by the number of struts in the examined section.

Preparation of Tissue Total Protein Extracts

All the extraction procedures were performed on ice with ice-cold reagents. Briefly, frozen porcine coronary arteries were crushed into a fine powder under liquid nitrogen and resuspended in an adequate volume of Cell Extraction Buffer containing 10 mmol/L Tris, pH 7.4;
2 mmol/L Na<sub>2</sub>VO<sub>4</sub>; 100 mmol/L NaCl; 1% Triton X-100; 1 mmol/L EDTA; 10% glycerol; 1 mmol/L EGTA; 0.1% SDS; 1 mmol/L NaF; 0.5% deoxycholate; 20 mmol/L Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, supplemented with 1 mmol/L phenylmethylsulfonyl fluoride and Protease Inhibitor Cocktail (P2714) (Sigma, Dorset, United Kingdom) just before use and then centrifuged for 15 minutes at 13000g. Supernatant was transferred to a fresh tube and stored at –80°C. Protein concentration was determined using the Bio-Rad protein assay kit.

**Western Blot Analysis**

The levels of proliferating cell nuclear antigen (PCNA) or CD68 expression were evaluated in total protein extracts from porcine coronary arteries 7 and 28 days after stent implantation respectively. Equivalent amounts of protein (60 μg) from each sample were electrophoresed on a 10% discontinuous polyacrylamide gel. The proteins were transferred onto nitrocellulose membranes according to the manufacturer’s instructions (Bio-Rad, Milan, Italy). The membranes were saturated by incubation with 10% milk buffer for 3 hours at room temperature and then incubated with mouse anti-PCNA antibody (1:3000, PC10, Sigma), mouse anti-CD68 (1:1000, AbD Serotec, Kidlington, United Kingdom), or mouse anti-β-actin antibody (1:5000, Sigma) overnight at 4°C. The membranes were washed 3 times with 0.5% Trition X-100 in PBS and then incubated with anti-mouse immunoglobulin coupled to peroxidase (1:2000, PerkinElmer, Monza, Italy) for 1 hour at room temperature. The immune complexes were visualized by enhanced chemiluminescence (Amersham ECL, GE Healthcare, Milan, Italy). ImageJ was used for densitometric analysis. Results are expressed as arbitrary units of PCNA or CD68 protein levels, normalized to protein levels of β-actin.

**Immunohistochemical Analysis**

After fixation of the coronary arteries harvested at day 7, the stent struts were gently removed with microforceps under a dissection microscope. The specimens were dehydrated, embedded in paraffin, and cut into 7-μm-thick slices. After antigen retrieval in citrate buffer, the sections were incubated with monoclonal mouse anti-PCNA antibody (1:250, PC10, Sigma) and biotinylated anti-mouse secondary antibody (1:200, DakoCytomation, Milan, Italy). Slides were treated with streptavidin–horseradish peroxidase (DakoCytomation) and exposed to diaminobenzidine chromogen (DakoCytomation) with hematoxylin counterstain. The proliferating cell number in the porcine coronary arteries was scored in 10 random fields (×20 objective) for 10 sections from each artery, under blind conditions, and expressed as the percentage of total arterial (medial plus neointimal) cells positive for PCNA 7 days after stent implantation.

**Cell Culture**

Porcine vascular SMCs were isolated from coronary arteries of male pigs as previously described<sup>25</sup> and grown in Dulbecco’s modified Eagle medium (Cambrex Bio Science, Walkersville, MD) supplemented with L-glutamine (Lonza, Treviglio, Italy), 10% fetal bovine serum (Lonza), 100 U/mL penicillin (Lonza), and 100 μg/mL streptomycin (Lonza) in a humidified incubator at 37°C in 5% CO<sub>2</sub>. Before initiation of assays, the SMCs were switched into Dulbecco’s modified Eagle’s medium supplemented with 0.1% fetal bovine serum for 48 hours, to achieve quiescence. Studies were performed with cells at passages 3 to 6.

**Proliferation Assay**

Cell proliferation was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. SMCs were plated on 48-well plastic culture plates at a density of 1.5×10<sup>4</sup> cells/well and then incubated with Dulbecco’s modified Eagle’s medium containing human tumor necrosis factor-α (TNF-α) (50 ng/mL, R&D Systems, Minneapolis, MN) for 72 hours in the presence or absence of bindarit (10–300 μmol/L). The absorbance values were obtained with an ELISA assay reader (630 nm).

**ELISA for MCP-1 Protein**

MCP-1 levels were measured in plasma samples obtained 28 days after stent implantation and in the SMC supernatants. SMCs plated as above were stimulated with human TNF-α (50 ng/mL) in the presence or absence of bindarit (10–300 μmol/L). After 12, 24, 48,
and 72 hours, media were collected and centrifuged at 2000g for 15 minutes at 4°C, and supernatants were used for ELISA according to the manufacturer’s instructions (Pig CCL-2 ELISA kit, Bethyl Laboratories, Montgomery, TX). The results are expressed as pg/mL.

**Statistical Analysis**

Results are expressed as mean±SEM of n animals for in vivo experiments and mean±SEM of multiple experiments for in vitro assays. The Student t test was used to compare 2 groups, and ANOVA (2-tailed probability value) was used with the Dunnett post hoc test for multiple groups using GraphPad Instat 3 software (San Diego, CA). A probability value of less that 0.05 was taken to indicate statistical significance.

**Results**

**Morphometric Analysis**

Representative stented artery sections obtained 28 days after implantation are shown in Figure 1. Morphometric assessment showed a significant reduction in neointimal area (4.03±0.46 versus 6.65±0.44 mm², P<0.001) (Figure 2A), neointimal thickness (270.2±26.92 versus 551.06±49.94 μm, P<0.001) (Figure 2B), percentage of stenosis (47.56±3.42% versus 74.97±2.71%, P<0.001) (Figure 2C), and inflammatory score (1.10±0.06 versus 1.83±0.11, P<0.001) (Figure 2D) in the bindarit-treated group compared with control animals. Moreover, in the bindarit-treated group, the lumen area was significantly increased compared with the control group (4.43±0.33 versus 2.12±0.15 mm², P<0.001) (Figure 2E). No significant differences were detectable in vessel area (10.48±0.56 versus 11.05±0.35 mm²), IEL area (8.47±0.50 versus 8.76±0.31 mm²), or medial area (2.01±0.08 versus 2.28±0.14 mm²). Importantly, there was no significant difference in the injury score (2.31±0.11 versus 2.23±0.13) (Figure 2F) and stent circumference (9.53±0.16 versus 9.55±0.28 mm²) between the 2 groups, indicating and that experimental and control animals had a similar degree of injury and complete stent deployment.

**Effect of Bindarit on In Vivo Proliferation**

Treatment with bindarit significantly reduced (by 45%, P<0.05) the number of PCNA-positive cells in the artery 7 days after stent implantation compared with control group (Figure 3A and 3B). Results were also confirmed by Western blot analysis (Supplemental Figure I, available online at http://atvb.ahajournals.org).

**Effect of Bindarit on Monocyte/Macrophage Infiltration**

Western blot analysis was performed to examine the effect of bindarit on the monocyte/macrophage infiltration. The monocyte/macrophage marker CD68 was highly expressed in coronary arteries 28 days after stent implantation. Bindarit significantly reduced (by 55%, P<0.01) CD68 levels as shown by relative densitometric analysis (Figure 3C and 3D).

**Effect of Bindarit on MCP-1 Plasma Levels**

A significant increase (P<0.01) in MCP-1 plasma concentration was observed in pigs subjected to stenting compared with the naïve animals (1903.05±172.64 pg/mL, n=9, versus 885.41±26.74 pg/mL, n=7). Bindarit caused a significant (P<0.05) inhibition of MCP-1 plasma levels at day 28 by ≈30% (1369.45±76.13 pg/mL, n=7) (Figure 4).
cells. When porcine SMCs were stimulated with TNF-α (50 ng/mL)–induced porcine smooth muscle cell proliferation. Results are expressed as mean ± SEM of 3 separate experiments run in triplicate. **P<0.01 vs unstimulated cells, *P<0.05 vs TNF-α.

Effect of Bindarit on Porcine SMC Proliferation

Initiation and maintenance of SMC proliferation is a critical event in the pathogenesis of intimal hyperplasia. As shown in Figure 5, bindarit at 100 and 300 μmol/L significantly inhibited TNF-α–induced porcine SMC proliferation by 33% and 50% (P<0.01, n=3), respectively. Cell viability (>95%) was not affected by bindarit at the concentrations used in this study (data not shown).

Effect of Bindarit on MCP-1 Production

To determine whether the in vitro antiproliferative effect of bindarit was associated with MCP-1 inhibition, MCP-1 protein concentration was determined by ELISA in the supernatants of cultured primary porcine SMCs. As shown in the Table, stimulation of SMCs with TNF-α (50 ng/mL) caused an increase in release of MCP-1 compared with unstimulated cells. When porcine SMCs were stimulated with TNF-α in the presence of bindarit (10–300 μmol/L), a significant inhibition of MCP-1 production was observed at 100 and 300 μmol/L.

Discussion

We previously demonstrated that bindarit inhibits neointimal formation in rodent models of vascular injury by a direct effect on SMC proliferation and migration and by reducing neointimal macrophage content; effects associated with the inhibition of MCP-1 production.17 However, although small animal models of neointimal formation have several advantageous characteristics (eg, low cost, ready availability, small size that limits the quantities of investigational drugs required for in vivo use), on many occasions they lack efficacy in predicting the success of interventions to inhibit restenosis in humans.18–20 Therefore, the aim of the present study was to evaluate the efficacy of bindarit in the preclinical model of in stent stenosis in pigs.

In this study, we have shown that bindarit given orally significantly reduces in-stent stenosis in the porcine coronary stent model. When compared with the controls, stented arteries from bindarit-treated animals showed a significant reduction of morphometric percentage stenosis area, from 75% to 47.5%, a decrease of 37%. Seventy-five percent of the stenosis area in the control group was a higher value than other reports in the literature.26,27 However, as shown by our injury score, no or minimal damage was induced to the media. According to the Gunn23 scoring system, an average value of ≈2.2 indicates an IEL deformed >45° in most of the samples analyzed in the absence of medial injury. Injury scores, external elastic lamina area, IEL area, and stent circumference were similar in both groups, confirming the homogeneity of the analyzed data in our model. In stented coronary arteries from animals treated with bindarit, neointimal area was significantly inhibited by 40% compared with control animals. Importantly, bindarit shows effects similar to those of paclitaxel- and sirolimus-coated stents on neointima formation in porcine models.22,27 A moderate inflammation was also observed in peri-stent areas, as assessed by the inflammatory score, and this was reduced by ≈40% in stented arteries from bindarit-treated animals.

Neointimal hyperplasia contributes to the development of in-stent restenosis,28 and a pivotal mechanism is the loss of differentiation of SMCs that become able to proliferate and migrate.29 It is well known that MCP-1 not only is a potent chemoattractant chemokine for monocytes/macrophages but may also directly induce SMC proliferation and migration through cell cycle proteins and intracellular proliferative signals.2–3 Interestingly, bindarit diminished the number of

Table. Effect of Bindarit on MCP-1 Production by TNF-α-Stimulated Porcine SMCs

<table>
<thead>
<tr>
<th>MCP-1 (pg/mL)</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated cells</td>
<td>242±32</td>
<td>1044±77</td>
<td>1906±150</td>
<td>2520±164</td>
</tr>
<tr>
<td>50 ng/mL TNF-α</td>
<td>1840±192*</td>
<td>3817±215*</td>
<td>5120±220*</td>
<td>5354±161*</td>
</tr>
<tr>
<td>50 ng/mL TNF-α+10 μmol/L bindarit</td>
<td>1637±123</td>
<td>3450±199</td>
<td>5450±310</td>
<td>6030±358</td>
</tr>
<tr>
<td>50 ng/mL TNF-α+30 μmol/L bindarit</td>
<td>1701±51</td>
<td>3920±257</td>
<td>4770±353</td>
<td>5389±266</td>
</tr>
<tr>
<td>50 ng/mL TNF-α+100 μmol/L bindarit</td>
<td>1241±55†</td>
<td>3046±152‡</td>
<td>4080±144‡</td>
<td>3782±116†</td>
</tr>
<tr>
<td>50 ng/mL TNF-α+300 μmol/L bindarit</td>
<td>1088±36‡</td>
<td>2430±133†</td>
<td>3400±174†</td>
<td>2824±110†</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM of 3 separate experiments run in triplicate. MCP indicates monocyte chemotactic protein; TNF, tumor necrosis factor; SMC, smooth muscle cell.

*P<0.01 vs unstimulated cells.
†P<0.01 vs TNF-α.
‡P<0.05 vs TNF-α.
arterial PCNA-positive proliferating cells 7 days after stent implantation and monocyte/macrophage content in injured vessels at 28 days, clearly showing either antiproliferative and antiinflammatory activity. These effects were associated with a significant inhibition of MCP-1 plasma levels. Increased levels of circulating MCP-1 in animals subjected to vascular injury are in keeping with an active role for this chemokine in tissue pathogenesis and correlate with epidemiological evidence showing higher MCP-1 plasma levels associated with human restenosis.30

Bindarit also displayed antiproliferative effects in vitro, with significant inhibition of TNF-α-induced porcine SMC proliferation. Furthermore, this was associated with a significant and concentration-related inhibition of MCP-1 amounts measured in the supernatants. These data are in agreement with our previous results on primary rat and mouse aortic SMCs.17

The present study has some limitations. Although the porcine coronary model seems to represent the human coronary artery response to stenting, mimicking several clinical conditions, including thrombosis and neointimal formation,20 it does not precisely simulate human in-stent restenosis.18,20 An important point in the present model is that stent implantation was performed in normal porcine coronary arteries, whereas in humans, much of the stent would be in contact with atheromatous plaque and not with media. Furthermore, in the present study the extent of in stent stenosis was examined only at 28 days after stent implantation; for example, longer follow-up should be performed to assess the effect of bindarit on arterial healing.

In conclusion, here we report the use of oral administration of bindarit as a viable approach to reduce in stent stenosis in pigs. Importantly, preclinical studies demonstrated that bindarit has a safe toxicological profile (rodent LD₅₀ ~2000 mg/kg PO and ~600 mg/kg IP) and is devoid of immuno-suppressive, mutagenic, and carcinogenic effects (Product Data Sheet, Angelini Research Center). Phase I clinical studies demonstrated that bindarit is well tolerated and confirmed the lack of overt toxicity suggested by preclinical studies (Product Data Sheet, Angelini Research Center). Results of Phase II clinical studies confirmed the good tolerability profile of bindarit and demonstrated, at 600 mg BID, significant effects in kidney disease patients.31,32 Currently, a double-blind, randomized, placebo-controlled clinical trial is ongoing (“The Effects of Bindarit in Preventing Stent Restenosis,” registered on ClinicalTrials.gov, identifier NCT01269242), with the aim of investigating the effect of bindarit in human coronary restenosis. Evidence of bindarit efficacy could provide clinicians with useful complementary or alternative therapeutic tools.

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Disclosures
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References


Figure I (A) Representative blot showing the effect of bindarit on PCNA expression in the protein extract of single coronary arteries 7 days after stenting. (B) Densitometric analysis of PCNA expression levels normalized to protein levels of β-actin. Data per single animal and means (bars) are presented. *$P<0.05$ vs control group.