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

Armando Ialenti, Gianluca Grassia, Peter Gordon, Marcella Maddaluno, Maria Vittoria Di Lauro, Andrew H. Baker, Angelo Guglielmotti, Antonio Colombo, Giuseppe Biondi, Simon Kennedy, Pasquale Maffia

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

Increasing evidence suggests that monocyte chemotactic protein (MCP)-1/CCL2 plays an early and important role in the formation of intimal hyperplasia and in-stent restenosis\(^1\) by increasing macrophage accumulation and smooth muscle cell (SMC) proliferation and migration.\(^2,3\) Deletion of the MCP-1 gene, blocking MCP-1 signaling or MCP-1 receptor CCR2 decreases neointimal hyperplasia after balloon- and stent-induced injury in several animal models.\(^4–7\) Similarly, catheter-based adenovirus-mediated anti-monocyte chemotactic gene therapy attenuates in-stent neointimal formation in monkeys.\(^8\) These data suggest that an antiinflammatory/antiproliferative strategy targeting MCP-1 might be an appropriate and reasonable approach for the prevention of neointimal formation and in-stent restenosis.

Bindarit is a selective inhibitor of MCP-1/CCL2, MCP-3/CCL7, and MCP-2/CCL8 synthesis\(^9\) that shows potent anti-inflammatory activity in a number of experimental models, including nephritis, arthritis, pancreatitis, and colitis,\(^10–13\) as well as reducing myocardial and renal dysfunction in swine renovascular hypertension.\(^14,15\) Phase II clinical trials have shown that bindarit is well tolerated and significantly reduced urinary MCP-1 and albumin excretion in kidney disease.\(^10,16\)

Interestingly, we have already shown that oral administration of bindarit is effective in reducing neointimal formation in both nonhyperlipidemic and hyperlipidemic 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/CCL2 pro-
duction.\textsuperscript{17} 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.\textsuperscript{18–20} 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.

\textbf{Methods}

\textbf{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.

\textbf{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, Aziende Chimiche Runiche Angieli 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 day follow-up period. 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\textsuperscript{14,15} and pharmacokinetic studies in male Göttingen minipigs (10–12 kg, Harlan) showing that bindarit is well absorbed when administered by the oral route. Single-dose oral administration of 25 mg/kg resulted in a $C_{\text{max}}$ level of 50 $\mu$g/mL (corresponding to 154 $\mu$mol/L), $T_{\text{max}}$ 2 hours, and t½ ~10 hours (Product Data Sheet, Angelini Research Center), corresponding to concentrations able to inhibit MCP-1 production and inflammation.\textsuperscript{14,15,17,21}

\textbf{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. Animals were sedated by an injection of tiletamine/zolazepam (100 mg Zoletil IM, Virbac, Suffolk, United Kingdom) and propofol (30 mg Rapinovet IV, Schering-Plough, Welwyn Garden City, United Kingdom). All animals were intubated and anesthesia maintained throughout the procedure using a mixture of isoflurane (1% to 2%, Abbott Laboratories Ltd, Maidenhead, UK) in oxygen/nitrous oxide. Unfractionated heparin (100 U/kg IV, Leo Laboratories, Princes Garden, 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 clopidogrel orally every 2 days for the duration of the study.

\textbf{Tissue Processing and Morphometric Analysis}

To determine whether bindarit was successful in reducing in-stent stenosis, stents from bindarit-treated animals and controls were examined histologically. One control animal failed to develop vascular injury in both implanted stents and was excluded from the study. Seven or 28 days after stent implantation, the pigs were euthanized with an overdose of pentobarbitone (200 mg/mL, pentobarbitone, Merial, Harlow, United Kingdom) via the marginal ear vein, and the stented arteries were removed from the heart and flushed with normal saline to remove nonadherent thrombus. The coronary arteries harvested 7 days after stent implantation were divided into 2 parts at the center of the stent. The proximal part was used for protein extraction, and the distal portion was fixed in 10% formaldehyde and used for immunohistochemical staining.

For morphometric analysis, stented coronary arteries harvested at 28 days were fixed in formal saline (24 hours) and dehydrated in pure acetone before resin embedding in glycol methacrylate (Technova, 7000, Kulzer, Wehrheim, Germany) following the manufacturer’s instructions. Sections (4–12 in controls and 5–12 in bindarit) were obtained from the proximal to distal portion of the stent using a Buehler Isomet 1000 rotary precision saw (Buehler, Lake Bluff, IL) and mounted on a glass slide. Sections were then ground and polished using a Buehler Metaserv 2000 grinder to reduce the thickness to 10 $\mu$m and give a uniform surface for staining and microscopic evaluation. Sections were stained using hematoxylin/eosin, and images were acquired using a Leica DM LB2 microscope and Leica DFC320 digital camera. After digitalizing, histomorphometric measurements were performed with ImageJ (NIH Imaging, http://rsbweb.nih.gov/ij/). Borders were manually traced for lumen area, area circumscribed by the internal elastic lamina (IEL), the border of the external elastic lamina (external elastic lamina area, vessel area) and stent circumference as the linear distance from strut to strut around the circumference of the stent.\textsuperscript{22} The neointimal and medial areas were computed as follows: neointimal area=IEL area minus lumen area; medial area=external elastic lamina area minus IEL area. Furthermore, percentage area of stenosis was calculated as 100\times1 (– lumen area/IEL area). Neointimal thickness (defined as the minimum distance between the strut and the lumen) was determined at each strut site and calculated as mean for each stented coronary segment. Results are expressed as the mean value from both stents per animal.

\textbf{Injury and Inflammatory Score}

The injury score was calculated as previously reported by Gunn et al.\textsuperscript{23} Briefly, we considered both deep injury and stretch as follows: 0=no impression of metal on media; 1=deformation of the IEL by $<45^\circ$; 2=deformation of the IEL by $>45^\circ$; 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.\textsuperscript{24} 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, circumferential lymphohistiocytic infiltrate surrounding the strut; 2=localized, moderate to dense cellular aggregate surrounding the strut noncircumferentially; 3=cytoplasmic density lymphohistiocytic 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.

\textbf{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% Triton 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, Treviglio, Italy), 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 × 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 2000 g 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 than 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 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 the protein extract of single coronary arteries 28 days after stenting. Bindarit inhibited (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=5). 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).
Effect of Bindarit on Porcine SMC Proliferation

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 restenosis in pigs.

In this study, we have shown that bindarit given orally significantly reduces in-stent restenosis 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.5,3 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 three 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.

Figure 5. Effect of bindarit (10–300 μmol/L) on tumor necrosis factor-α (TNF-α)-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<0.01 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. 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 (up to a dose of 1200 mg BID) 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. 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


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Supplemental Material

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.
MCP 억제제인 Bindarit은 스텐트 재협착을 막는다.

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한림대학교 성심병원 순환기내과

Summary

배경
Bindarit은 단핵구 주화성 단백질(monocyte chemotactic protein, MCP)을 선택적으로 억제한다. Bindarit은 설치류의 혈관손상 모델에서 혈관의 평활근세포의 증식과 이동, 신생내막의 대식세포를 감소시킴으로써 신생내막 형성을 효과적으로 억제시킨다는 보고가 있다. 본 연구는 bindarit의 돼지 관상동맥 스텐트 모델에서 스텐트 내 협착에 대한 효과를 평가하고 있다.

방법 및 결과
1 또는 2개의 금속 스텐트(Multi-Link Vision, 3.5mm)를 42마리의 돼지 관상동맥에 삽입하였다(1:1.2의 동맥 대 스텐트 사이즈 비율, bindarit군 20마리, 대조군 22마리). Bindarit을 50mg/kg/day로 스텐트 삽입 2일전부터 7일에서 28일 사이의 사망 때까지 경구로 복용시켰다. Bindarit은 신생내막 부위를 의미 있게 감소시켰고(39.4%, P<0.001, n=9 group), 신생내막 두께(51%, P<0.001)와 협착 부위의 넓이(37%, P<0.001), 염증 지수(40%, P<0.001)도 대조군 동물에 비해 의미 있게 감소시켰다. 반면에 양군에서 혈관손상 지표에서는 차이가 없었다. 더욱이 bindarit 치료는 스텐트 삽입 부위에서 증식세포의 수와(by 45%, P<0.05; n=6 group), monocyte/macrophage를 7일, 28일째에 의미 있게 감소시켰다(by 55%, P<0.01; n=5-6 group). 이러한 효과는 MCP-1의 28일째 혈청 농도의 의미 있는 감소와 연관이 있었다(P<0.05). In vitro 연구에서 bindarit 10-300μmol/L은 tumor necrosis factor-α (50ng/mL)에 의해 유발된 돼지의 관상동맥 평활근세포의 증식을 감소시키고 MCP-1 생산을 억제시켰다.

결론
Bindarit은 돼지 관상동맥의 스텐트 내 협착을 예방하는 효과가 있어서 향후 임상적응을 위한 연구의 근거를 제공한다.
Commentary


본 연구의 주된 결과는 bindarit을 경구로 복용했을 때 관상동맥의 금속 스텐트의 재협착을 의미 있게 감소시켰다는 점이다. 즉 협착을 37% 감소시키고 신생내막의 넓이를 40% 감소시켰는데 이는 이전의 돼지 동물실험에서 paclitaxel- 혹은 sirolimus-coated stents와 비슷한 정도의 감소 효과를 보이고 있어서 매우 흥미로운 결과이다(Figure 1).

MCP-1은 monocyte나 macrophage에 대한 강력한chemoattractant이지만 직접적으로 혈관 평활근세포의 증식과 이동을 유도하는데 대개 cell cycle 단백질과 세포 내의 증식 신호를 통해서라고 알려져 있다.

또한 bindarit은 MCP-1을 억제뿐만 아니라 TNG-α에 의해서도 혈관 평활근세포 증식을 억제한다. 현재 주로 사용되고 있는 약물 방출 스텐트가 비록 신생내막 증식 억제에는 효과적이지만 지속적으로 스텐트 혈전증의 위험에 노출되게 한다는 점을 생각한다면 본 연구결과는 매우 고무적이다. 특히 수술이나 출혈의 위험이 높은 고연령의 환자군에서 허혈성 심질환이 있는 경우에 일반 금속 스�enstein와 bindarit의 조합이 매우 유용할 수 있었다. 현재 임상시험(The Effects of Bindarit in...
Preventing Stent Restenosis이 진행 중에 있으므로 좋은 결과가 나온다면 임상의에게 또 다른 관상동맥 혈착증 치료도구를 제시해줄 수 있겠다.

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

Armando Ialenti, Gianluca Grassia, Peter Gordon, Marcella Maddaluno, Maria Vittoria Di Lauro, Andrew H. Baker, Angelo Guglielmotti, Antonio Colombo, Giuseppe Biondi, Simon Kennedy, Pasquale Maffia

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

Increasing evidence suggests that monocyte chemotactic protein (MCP)-1/CCL2 plays an early and important role in the formation of intimal hyperplasia and in-stent restenosis\(^1\) by increasing macrophage accumulation and smooth muscle cell (SMC) proliferation and migration.\(^2,3\) Deletion of the MCP-1 gene, blocking MCP-1 signaling or MCP-1 receptor CCR2 decreases neointimal hyperplasia after balloon- and stent-induced injury in several animal models.\(^4–7\) Similarly, catheter-based adenovirus-mediated anti-monocyte chemotactrant gene therapy attenuates in-stent neointimal formation in monkeys.\(^8\) These data suggest that an antiinflammatory/antiproliferative strategy targeting MCP-1 might be an appropriate and reasonable approach for the prevention of neointimal formation and in-stent restenosis.

Bindarit is a selective inhibitor of MCP-1/CCL2, MCP-3/CCL7, and MCP-2/CCL8 synthesis\(^9\) that shows potent anti-inflammatory activity in a number of experimental models, including nephritis, arthritis, pancreatitis, and colitis,\(^10–13\) as well as reducing myocardial and renal dysfunction in swine renovascular hypertension.\(^14,15\) Phase II clinical trials have shown that bindarit is well tolerated and significantly reduced urinary MCP-1 and albumin excretion in kidney disease.\(^10,16\)

Interestingly, we have already shown that oral administration of bindarit is effective in reducing neointimal formation in both nonhyperlipidemic and hyperlipidemic 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/CCL2 pro-
duction. Therefore, 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, Aziende Chimiche Riunite 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 day follow-up period. 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 Göttingen minipigs (10–12 kg, Harlan) showing that bindarit is well absorbed when administered by the oral route. Single-dose oral administration of 25 mg/kg resulted in a Cmax level of 50 μg/mL (corresponding to 154 μmol/L). T1/2 was 2 hours, and T1/2 was 10 hours (Product Data Sheet, Angelini Research Center), corresponding to concentrations able to inhibit MCP-1 production and inflammation.

**Porcine Coronary Stent Model**

Pigs were premedicated with aspirin (150 mg oral, Teva, Leeds, United Kingdom) and clopidogrel (150 mg oral, Sanofi-Aventis, Coventry, United Kingdom) over a 24-hour period before surgery. Pigs were sedated by an injection of tiletamine/zolazepam (100 mg Zoletil IM, Virbac, Suffolk, United Kingdom) and propofol (30 mg Rapinovet IV, Schering-Plough, Welwyn Garden City, United Kingdom). All animals were intubated and anesthesia maintained throughout the procedure using a mixture of isoflurane (1% to 2%, Abbott Laboratories Ltd., Maidenhead, UK) in oxygen/nitrous oxide. Unfractionated heparin (100 U/kg IV, Leo Laboratories, Princes Risborough, United Kingdom) was given at the start of the procedure. Access to the coronary arteries was achieved via the left femoral artery, using standard 6 French sheaths and coronary guiding catheters. In the majority of animals, stents (Multi-Link Vision, 3.5x15 mm, Abbott) were placed in 2 of the 3 coronary arteries (left anterior descending, left circumflex, and right coronary arteries) under fluoroscopic guidance. Nine bindarit-treated animals (16 stents) and 10 control animals (17 stents) were used for the morphometric analysis. Six bindarit-treated animals (12 stents) and 6 control animals (12 stents) were used for the proliferating cell nuclear morphometric analysis. Six bindarit-treated animals (12 stents) and 6 control animals (12 stents) were used for antigen analysis. Five bindarit-treated animals (10 stents) and 6 control animals (12 stents) were used for the proliferating cell nuclear morphometric analysis. Six bindarit-treated animals (12 stents) and 6 control animals (12 stents) were used for the proliferating cell nuclear morphometric analysis.

**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; normal diet, with supplementation of 75 mg of aspirin orally every 2 days and 75 mg of clopidogrel orally every 2 days for the duration of the study.

**Tissue Processing and Morphometric Analysis**

To determine whether bindarit was successful in reducing in-stent stenosis, stents from bindarit-treated animals and controls were examined histologically. One control animal failed to develop vascular injury in both implanted stents and was excluded from the study. Seven or 28 days after stent implantation, the pigs were euthanized with an overdose of pentobarbital (200 mg/mL pentobarbitone, Merial, Harlow, United Kingdom) via the marginal ear vein, and the stented arteries were removed from the heart and flushed with normal saline to remove nonadherent thrombus. The coronary arteries harvested 7 days after stent implantation were divided into 2 parts at the center of the stent. The proximal part was used for protein extraction, and the distal portion was fixed in 10% formaldehyde and used for immunohistochemical staining.

For morphometric analysis, stented coronary arteries harvested at 28 days were fixed in formalin saline (24 hours) and dehydrated in pure acetone before resin embedding in glycol methacrylate (Technovit 8100, Kulzer, Wehrheim, Germany) following the manufacturer’s instructions. Sections (4–12 in controls and 5–12 in bindarit) were obtained from the proximal to distal portion of the stent using a Buehler Isomet 1000 rotary precision saw (Buehler, Lake Bluff, IL) and mounted on a glass slide. Sections were then ground and polished using a Buehler Metaserv 2000 grinder to reduce the thickness to 10 μm and give a uniform surface for staining and microscopic evaluation. Sections were stained using hematoxylin/eosin, and images were acquired using a Leica DM LB2 microscope and Leica DFC320 digital camera. After digitizing, histomorphometric measurements were performed with ImageJ (NIH Imaging, http://rsbweb.nih.gov/ij). Borders were manually traced for lumen area, area circumscribed by the internal elastic lamina (IEL), the border of the external elastic lamina (external elastic lamina area, vessel area) and stent circumference as the linear distance from strut to strut around the circumference of the stent. The neointimal and medial areas were computed as follows: neointimal area = IEL area minus lumen area; medial area = external elastic lamina area minus IEL area. Furthermore, percentage area of stenosis was calculated as 100×(1−lumen area/IEL area). Neointimal thickness (defined as the minimum distance between the strut and the lumen) was determined at each strut site and calculated as mean for each stented coronary segment. Results are expressed as the mean value from both stents per animal.

**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°; 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, noncircumferential 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.

**ECG and mean arterial pressure**

After coronary stent deployment, ECG and arterial pressure were recorded continuously. Stents were deployed at inflation pressures necessary to produce a stent to artery ratio of 1.2:1. After sheath removal, the femoral artery was ligated, and the leg wound was closed and sutured. All animals were given buprenorphine (0.15 mg of Vetgesic IM, Alstoe Ltd, York, United Kingdom) to provide analgesia and amoxicillin (350 mg of Amfipen LA IM, Intervet, Welwyn Garden City, United Kingdom) for antibiotic cover, immediately after the procedure. Animals were recovered and received a
2 mmol/L Na₃VO₄; 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 Na₄P₂O₇, 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% Triton 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 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, Treviglio, Italy), 100 U/mL penicillin (Lonza), and 100 μg/mL streptomycin (Lonza) in a humidified incubator at 37°C in 5% CO₂. 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 1.5×10⁴ 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 2000 g 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 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 = 5). 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).
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. However, although small animal models of neointimal formation have several advantageous characteristics (e.g., 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. 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. However, as shown by our injury score, no or minimal damage was induced to the media. According to the Gunn 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. 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 and a pivotal mechanism is the loss of differentiation of SMCs that become able to proliferate and migrate. 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. Interestingly, bindarit diminished the number of

![Figure 5. Effect of bindarit (10–300 μmol/L) on tumor necrosis factor-α (TNF-α)-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<0.01 vs TNF-α.](image-url)

**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 three 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.20

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 immunosuppressive, mutagenic, and carcinogenic effects (Product Data Sheet, Angelini Research Center). Phase I clinical studies demonstrated that bindarit (up to a dose of 1200 mg BID) 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


