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Coordination of lapachol to bismuth(III) improves its anti-inflammatory and anti-angiogenic activities

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Abstract Complex $[Bi(Lp)_2]Cl$ was obtained with 4-hydroxy-3-(3-methylbut-2-enyl)naphthalene-1,2-dione, "lapachol" (HLp). Lapachol, $[Bi(Lp)_2]Cl$ and $BiCl_3$ were evaluated in a murine model of inflammatory angiogenesis induced by subcutaneous implantation of polyether polyurethane sponge discs. Intraperitoneal (i.p.) administration of lapachol or $[Bi(Lp)_2]Cl$ reduced the hemoglobin content in the implants suggesting that reduction of neo-vascularization was caused by lapachol. In the *per os* treatment only $[Bi(Lp)_2]Cl$ decreased the hemoglobin content in the implants. Likewise, *N*-acetylglucosaminidase (NAG) activity decreased in the implants of the groups i.p. treated with lapachol and $[Bi(Lp)_2]Cl$ while in the *per os* treatment inhibition was observed only for $[Bi(Lp)_2]Cl$. Histological analysis showed that the components of the fibro-vascular tissue (vascularization and inflammatory cell population) were decreased in lapachol- and complex-treated groups. Our results suggest that both lapachol and $[Bi(Lp)_2]Cl$ exhibit anti-angiogenic and anti-inflammatory activities which have been attributed to the presence of the lapachol ligand. However, coordination to bismuth(III) could be an interesting strategy for improvement of lapachol's therapeutic properties.

Keywords Lapachol · Bismuth(III) complex · Sponge implants · Angiogenesis · Inflammation

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Introduction

Inflammation, angiogenesis and remodeling are self-limiting processes under normal healing conditions (Mendes et al. 2009a). Angiogenesis is a fundamental process to normal and abnormal tissue growth and repair, which consists of recruiting endothelial cells toward an angiogenic stimulus. However, if one or more of those processes are maintained further injury is caused resulting in chronic inflammatory conditions (Gong and Koh 2010; Mendes et al. 2009a; Walsh and Pearson 2001). Chronic inflammatory processes such as rheumatoid arthritis, Crohn's disease and psoriasis share these abnormal healing features. Thus, therapies that attenuate inflammatory angiogenesis and fibrotic processes are able to prevent progression and/or maintenance of chronic inflammatory conditions (Araújo et al. 2010; Xavier et al. 2010).

4-Hydroxy-3-(3-methylbut-2-enyl)naphthalene-1,2-dione, "lapachol" (Fig. 1), is a naphthoquinone

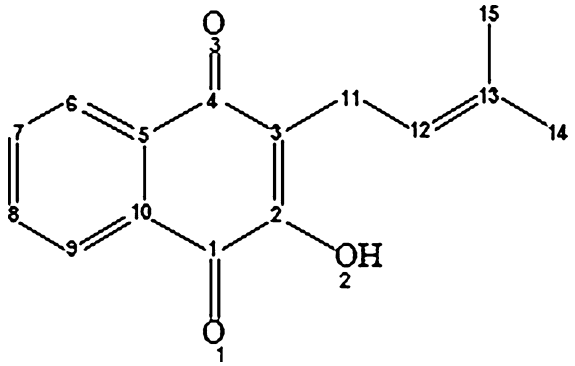

Fig. 1 Structure of 4-hydroxy-3-(3-methylbut-2-enyl)naphthalene-1,2-dione (Lapachol)

obtained from the heartwood of Brazilian *Tabebuia* trees (Bignoniaceae family) (Da Silva Júnior et al. 2011), which exhibits wide spectrum of biological activities, such as antitumoral, antimicrobial and antiprotozoal activities (Eyong et al. 2008; Oliveira et al. 2010). Anti-inflammatory activity was also demonstrated in studies with animal models (De Almeida et al. 1990; Lira et al. 2008).

Bismuth has long been associated with Medicine. The first full account of the internal administration of a bismuth compound was in 1786 by Louis Odier for the treatment of dyspepsia. Currently, the major medicinal use of bismuth compounds is for treating gastrointestinal disorders (Briand and Burford 1999; Yang and Sun 2007). The roles of these compounds in gastric and duodenal ulcer therapy and the eradication of *Helicobacter pylori*—a bacterium associated with the pathogenesis of gastro-duodenal ulcers—have been extensively investigated (Sadler et al. 1999; Severi et al. 2009). Pharmacological studies suggest that the treatment and prevention of ulcers by colloidal bismuth subcitrate (CBS) involve antimicrobial action together with fortification of gastric mucus and the stimulation of cytoprotective processes (Hall 1989; Lee 1991). Besides bismuth's activities on gastrointestinal disorders, investigations on the anti-inflammatory activity of bismuth subgallate (BSG) have been reported (Lin et al. 2004). Results suggest that BSG has an effect on suppressing nitric oxide and prostaglandin E_2, important mediators in inflammatory processes.

In the present work we studied the effects of lapachol (HLp), its bismuth(III) complex $[Bi(Lp)_2]Cl$ and $BiCl_3$ in an experimental model of inflammatory angiogenesis induced by a sponge implant in order to characterize probable synergistic effects between the metal and the naphthoquinone on murine models. The effects of the compounds on the angiogenic and inflammatory components of the fibro-vascular tissue were investigated.

Experimental

Materials and methods

Partial elemental analyses were performed on a Perkin Elmer CHN 2400 analyzer. An YSI model 31 conductivity bridge was employed for molar conductivity measurements. Infrared spectra were recorded on a Perkin Elmer FT-IR Spectrum GX spectrometer using KBr plates (4000–400 cm^{-1}) and Nujol mulls between CsI plates (400–200 cm^{-1}). NMR spectra were obtained with a Bruker DPX-200 Avance (200 MHz) spectrometer using DMSO-d_6 as the solvent and TMS as internal reference.

All common chemicals were purchased from Aldrich and used without further purification.

Isolation of lapachol

Lapachol was isolated from *Tabebuia* sp. using a method described by Ferreira (1996). The sawdust of *Tabebuia* sp. wood (100 g) was treated with 300 ml of 10% Na_2CO_3 aqueous solution, and maintained under stirring during 5 min. The red solution was filtered and the residue submitted to another extraction using 400 ml of sodium carbonate solution, and stirred for 10 min. The filtrate was carefully treated with concentrated HCl, drop by drop with constant agitation, until pH < 5, which is easily identified by the change of color from red to yellow. The extraction mixture was stirred for 30 min, and filtered in a Büchner funnel, under vacuum. After dried, the obtained solid material was washed with cold distilled water. The yellowish solid material (900 mg) was submitted to silica gel (70–230 Mesh, Merck) column chromatography, eluted with chloroform. The obtained yellow solid was re-crystallized with hexane to give pure yellow crystals (350 mg). The yellow crystalline material was chemically identified as lapachol through comparison with authentic sample using thin layer chromatography

and mixed melting point. Melting point: 139–141°C. IR (cm^{-1}): 3352 ν(OH), 1661 and 1639 ν(C=O), 1450–1591 ν(C=C); ^{1}H NMR (δppm) 1.68 and 1.79 (s, 2 Me), 3.3 (d, CH_2–CH=) and 5.2 (t, CH_2–CH=).

Synthesis of the bismuth(III) complex

The bismuth(III) complex was obtained by stirring under reflux an ethanol solution of lapachol (HLp) with bismuth chloride, $BiCl_3$, together with sodium acetate, $NaCH_3COO$, in 3:1:3 lapachol:$BiCl_3$:$NaCH_3COO$ molar ratio. The obtained solid was filtered off, washed with ethanol and diethylether and then dried.

[Bi(Lp)$_2$]Cl

Orange solid. Anal. Calc. for $C_{30}H_{26}O_6ClBi$ (726.96 g mol^{-1}): C 49.57%; H 3.60%. Found: C 49.01%; H 3.60%. Molar conductivity (1×10^{-3} mol l^{-1} DMF): 77.35 Ω^{-1} cm^2 mol^{-1}. IR (KBr, cm^{-1}): ν(C=O) 1634, 1623, ω(C–H) 729. IR (CsI/Nujol, cm^{-1}): ν(M–O) 489, 472. Signals in ^{1}H NMR (DMSO-d_6): δ (ppm) = 7.87 (1H, H9), 7.80 (1H, H6), 7.71 (1H, H8), 7.57 (1H, H7), 5.11 (1H, H12), 3.10 (2H, H11), 1.65 (3H, H14), 1.51 (3H, C15). Yield: 77%.

Anti-angiogenic activity

Animals

Male Swiss mice 7–8 weeks (20–30 g body weight) were used in these experiments. The mice were provided by the Central Animal Facility at the Institute of Biological Sciences, Federal University of Minas Gerais, Brazil. The animals were housed individually and provided with chow pellets and water ad libitum. The light/dark cycle was 12:12 h with lights on at 7:00 a.m. and lights off at 7:00 p.m. Efforts were made to avoid all unnecessary distress to the animals. Housing, anaesthesia and post-operative care concurred with the guidelines established by our local Institutional Animal Welfare Committee.

Preparation of sponge discs and implantation

Polyether–polyurethane sponge (Vitafoam Ltd., Manchester, UK) was used as the implanted material. The implants were discs, 5 mm thick × 8 mm diameter and were soaked overnight in 70% v/v ethanol and sterilized by boiling in distilled water for 15 min before implantation. For that, the animals were anaesthetized with 2,2,2-tribromoethanol (1 mg kg^{-1}; i.p. Aldrich, USA), the dorsal hair shaved and the skin wiped with 70% ethanol. The sponge discs were aseptically implanted into a subcutaneous pouch, which had been made with curved artery forceps through a 1 cm long dorsal mid-line incision. Post-operatively, the animals were monitored for any signs of infection at the operative site, discomfort or distress; any showing such signs were immediately humanely killed.

Intraperitoneal (i.p.) and per os administrations of the compounds

Suspensions of lapachol, $BiCl_3$ and bismuth(III) complex [Bi(Lp)$_2$]Cl were prepared in tween 80.6% in saline (i.p. route) and in carboxymethylcellulose (CMC) 0.5% in saline (*per os* route). Lapachol 25 mg kg^{-1} day^{-1} was used as positive control (Bezerra et al. 2008). Doses of $BiCl_3$ (16.3 mg kg^{-1} day^{-1}) and [Bi(Lp)$_2$]Cl (37.7 mg kg^{-1} day^{-1}) were equimolar to lapachol 25 mg kg^{-1} day^{-1}. In *per os* treatment, doses of the compounds ten-fold less were also used to show dose response effects. Higher doses were not used since high doses of lapachol (80 and 100 mg kg^{-1}) administered orally present toxic effects (Maeda et al. 2008).

Treatments started on the day of sponge implantation and finished after 8 days. Control groups received vehicles in the same schedule. The treatment was well tolerated by the animals over the experimental period.

Tissue extraction and determination of myeloperoxidase and N-acetylglucosaminidase activities

The number of neutrophils in implants was measured by assaying myeloperoxidase (MPO) activity as previously described (Ferreira et al.2004; Mendes et al. 2009a). The implants for this set of experiments were removed 9 days post-implantation after daily doses of the compounds. After excision they were weighed, homogenized in pH 4.7 buffer (0.1 mol l^{-1} NaCl, 0.02 mol l^{-1} $NaPO_4$, 0.015 mol l^{-1} NaEDTA), centrifuged at 12,000×g for 10 min. The pellets were then re-suspended in 0.05 mol l^{-1} $NaPO_4$

buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) followed by three freeze–thaw cycles using liquid nitrogen. MPO activity in the supernatant samples was assayed by measuring the change in absorbance (optical density; OD) at 450 nm using tetramethylbenzidine (1.6×10^{-3} mol l^{-1}) and H_2O_2 (0.3×10^{-3} mol l^{-1}). The reaction was terminated by the addition of 50 µl of H_2SO_4 (4 mol l^{-1}). Results were expressed as change in OD per gram of wet tissue.

The infiltration of mononuclear cells into the implants was quantified by measuring the levels of the lysosomal enzyme *N*-acetylglucosaminidase (NAG) present in high levels in activated macrophages (Ferreira et al. 2004; Mendes et al. 2009a). The implants removed 9 days post-implantation were homogenized in NaCl solution (0.9% w/v) containing 0.1% v/v Triton X-100 (Promega) and centrifuged ($3{,}000 \times g$; 10 min at 4°C). Samples (100 µl) of the resulting supernatant were incubated for 10 min with 100 µl of *p*-nitrophenyl-*N*-acetyl-beta-D-glucosaminide (Sigma) prepared in citrate/phosphate buffer (0.1 mol l^{-1} citric acid, 0.1 mol l^{-1} Na_2HPO_4; pH 4.5) to yield a final concentration of 2.24×10^{-3} mol l^{-1}. The reaction was stopped by the addition of 100 µl of 0.2 mol l^{-1} glycine buffer (pH 10.6). Hydrolysis of the substrate was determined by measuring the absorption at 400 nm. Results were expressed as nmol mg^{-1} wet tissue.

Hemoglobin extraction

The extent of vascularization of the sponge implants was assessed by the amount of hemoglobin (Hb) detected in the tissue using the Drabkin method (Ferreira et al. 2004; Mendes et al. 2009a). At day 9 post-implantation, the animals were killed and the sponge implants carefully removed, dissected free from adherent tissue and weighed. Each implant was homogenized (Tekmar TR-10, OH) in 5 ml of Drabkin reagent (Labtest, São Paulo, Brazil) and centrifuged at $12{,}000 \times g$ for 20 min. The supernatants were filtered through a 0.22 µm Millipore filter. The hemoglobin concentration in the samples was determined spectrophotometrically by measuring absorbance at 540 nm using an ELISA plate reader and compared against a standard curve of hemoglobin. The content of hemoglobin in the implant was expressed as µg Hb per mg wet tissue.

Histological analysis and staining

The sponge implants from separate groups of mice were excised carefully, dissected free of adherent tissue and fixed in formalin (10% w/v in isotonic saline). Sections (5 µm) were stained with hematoxylin and eosin (H&E) and processed for light-microscopic studies.

Statistical analysis

All data were expressed as mean ± SEM. Comparisons between the three groups (numbers as stated in the Figure legends; usually 5 to 13 animals) were made using one-way analysis of variance (ANOVA) followed by Newman–Keuls correction factor for multiple comparisons as a post-test. Differences between means were considered significant when P values were <0.05.

Results and discussion

Formation of the bismuth(III) complex

Microanalyses and molar conductivity data are compatible with the formation of $[Bi(L)_2]Cl$, in which two lapacholate anions are attached to the metal center.

Spectroscopic characterization

The band localized at 3352 cm^{-1} assigned to ν(O–H) in the infrared spectrum of free lapachol disappears upon coordination, indicating deprotonation. The characteristic ν(C=O) absorptions, found at 1661 and 1639 cm^{-1} in the spectrum of lapachol (Caruso et al. 2009; Martínez et al. 2003), shift to lower frequencies in the complex (1634 and 1623 cm^{-1}), in agreement with coordination through the oxygen atoms. The vibration attributed to an out-of-plane ring mode, ω(C–H), at 724 cm^{-1} in the infrared spectrum of lapachol shifts to 729 cm^{-1} in the spectrum of the complex (Caruso et al. 2009). New bands at 489 and 472 cm^{-1} were attributed to the ν(M–O) stretching vibration (Nakamoto 1970).

The ^{1}H NMR spectra of lapachol and its bismuth(III) complex $[Bi(Lp)_2]Cl$ were recorded in DMSO-d_6. The ^{1}H resonances were assigned on the

basis of chemical shifts and multiplicities. The ^{13}C NMR spectrum of the complex was not obtained due to its low solubility.

In the ^{1}H NMR spectrum of the complex the signals of all hydrogen atoms undergo shifts in relation to their position in free lapachol. However, the most significant shifts were observed for the aromatic hydrogens, suggesting coordination through the O(1) and O(2) oxygen atoms.

Measurement of the anti-angiogenic effect

In the i.p. treatment, the hemoglobin levels in the implants from vehicle-treated animals were 0.79 ± 0.18 μgHb mg^{-1} wet tissue. Lapachol and the complex statistically reduced neo-vascularization of the implants, as detected by changes in the hemoglobin content (Fig. 2a). In both cases decreasing on hemoglobin content was approximately 50%. Since lapachol and complex groups did not present statistical difference between each other, results suggest that the reduction of neo-vascularization is caused by free lapachol in both experimental groups. Thus, the complex probably dissociates under in vivo conditions releasing lapachol molecules after i.p. administration. Decreasing on hemoglobin values was not observed in the implants from animals treated with $BiCl_3$.

Hemoglobin levels in the implants from *per os* vehicle-treated animals were 0.72 ± 0.06 μgHb mg^{-1} wet tissue (lapachol dose of 25 mg kg^{-1} day^{-1}). After *per os* treatment with lapachol 25 mg kg^{-1} day^{-1} and equivalent doses of $BiCl_3$ and $[Bi(Lp)_2]Cl$ only complex $[Bi(Lp)_2]Cl$ statistically decreased the hemoglobin content in the implants (about 60%). Lapachol and $BiCl_3$ groups were unable to reduce neo-vascularization of the implants (Fig. 2b). *Per os* treatment was also performed with lapachol 2.5 mg kg^{-1} day^{-1} and equivalent doses of $BiCl_3$ and $[Bi(Lp)_2]Cl$ to evaluate dose response effects (Fig. 2c). In this case no statistical difference was observed among the hemoglobin levels of the treated and control groups.

The discrepancy between results of i.p. and *per os* treatments may well be due to the distinct administration models. *Per os* administration comprises a great number of variables, such as rate of dissolution of the compounds and low gastric pH. Since these factors influence the absorption and bioavailability of the compounds, they may have been responsible for the loss of activity of lapachol. Thus, results suggest that coordination seems to be a good strategy to

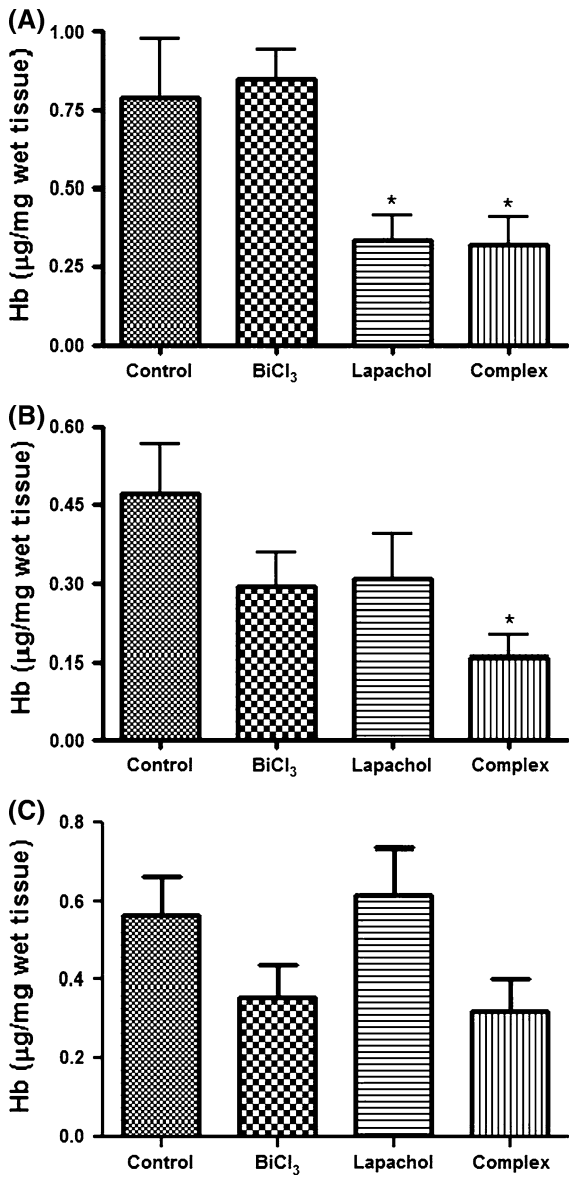

Fig. 2 Effects in the hemoglobin content induced by lapachol, complex $[Bi(Lp)_2]Cl$ and $BiCl_3$ on angiogenesis in murine models (n = 6–8) for **a** i.p. treatment with lapachol at 25 mg kg^{-1} day^{-1} and equivalent doses of $BiCl_3$ and bismuth(III) complex, **b** *per os* treatment with lapachol at 25 mg kg^{-1} day^{-1} and equivalent doses of $BiCl_3$ and bismuth(III) complex, and **c** *per os* treatment with lapachol at 2.5 mg kg^{-1} day^{-1} and equivalent doses of $BiCl_3$ and bismuth(III) complex. *statistically different in comparison to control, $P < 0.05$

improve the anti-angiogenic profile of lapachol in the *per os* treatment.

Measurement of leukocyte accumulation

Inflammatory components of the sponge-induced chronic inflammation were determined by estimating the number of leukocytes in the implant by assaying marker enzyme activities. Macrophage and neutrophils accumulation in the implants were assessed by measuring NAG and MPO activities (Mendes et al. 2009b).

In the i.p. treatment, NAG activity was statistically decreased in the treated groups with lapachol and complex $[Bi(Lp)_2]Cl$, showing an effect in this inflammatory cell population. After *per os* treatment with lapachol 25 mg kg^{-1} day^{-1} and equivalent doses of $BiCl_3$ and $[Bi(Lp)_2]Cl$, NAG activity was inhibited relative to the control group only by the bismuth(III) complex. Thus, it is clear that lapachol was not able to interfere with macrophages recruitment after *per os* administration. Interestingly, the obtained results are in agreement with the reduction of neo-vascularization, where coordination to bismuth(III) seemed to improve the activity of lapachol in the *per os* treatment.

In the *per os* treatment with using lapachol 2.5 mg kg^{-1} day^{-1} and equivalent doses of $BiCl_3$ and $[Bi(Lp)_2]Cl$ no statistical difference was observed between NAG activity in the treated and control groups (Fig. 3).

In both i.p. and *per os* treatments, MPO activity in the implants did not decrease suggesting that the studied compounds were unable to reduce neutrophils recruitment/activation in this model of inflammatory angiogenesis.

Gross appearance of implants and histological assessments

No signs of infection or rejection were observed in the implant location or in the incision during the 9-day period of the experiment. Histological analysis (H&E) showed that this procedure induced a fibro-vascular response causing the synthetic sponge matrix to be filled with a newly formed tissue (Fig. 4). The control implants were infiltrated by fibro-vascular stroma occupying the entire sponge by day 9. The tissue was composed of a dense inflammatory infiltrate with various cell types such as

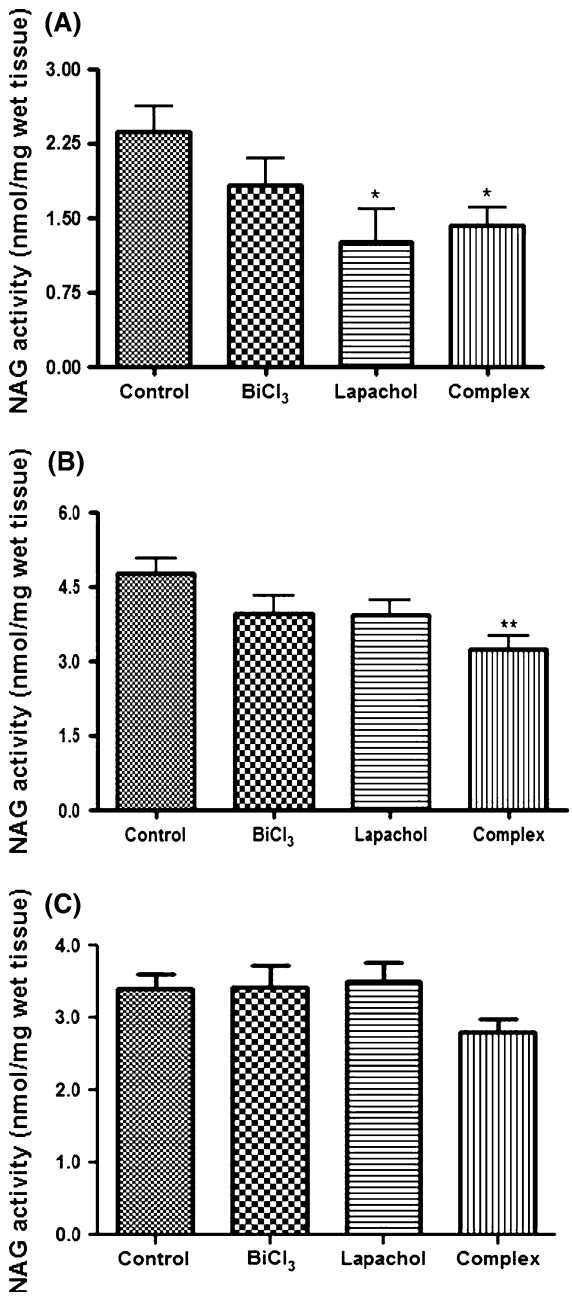

Fig. 3 Effects in the macrophage accumulation induced by lapachol, complex $[Bi(Lp)_2]Cl$ and $BiCl_3$ on angiogenesis in murine models (n = 8–13) for **a** i.p. treatment with lapachol at 25 mg kg^{-1} day^{-1} and equivalent doses of $BiCl_3$ and bismuth(III) complex, **b** *per os* treatment with lapachol at 25 mg kg^{-1} day^{-1} and equivalent doses of $BiCl_3$ and bismuth(III) complex, and **c** *per os* treatment with lapachol at 2.5 mg kg^{-1} day^{-1} and equivalent doses of $BiCl_3$ and bismuth(III) complex. * and **statistically different in comparison to control, $P < 0.05$ and $P < 0.01$, respectively

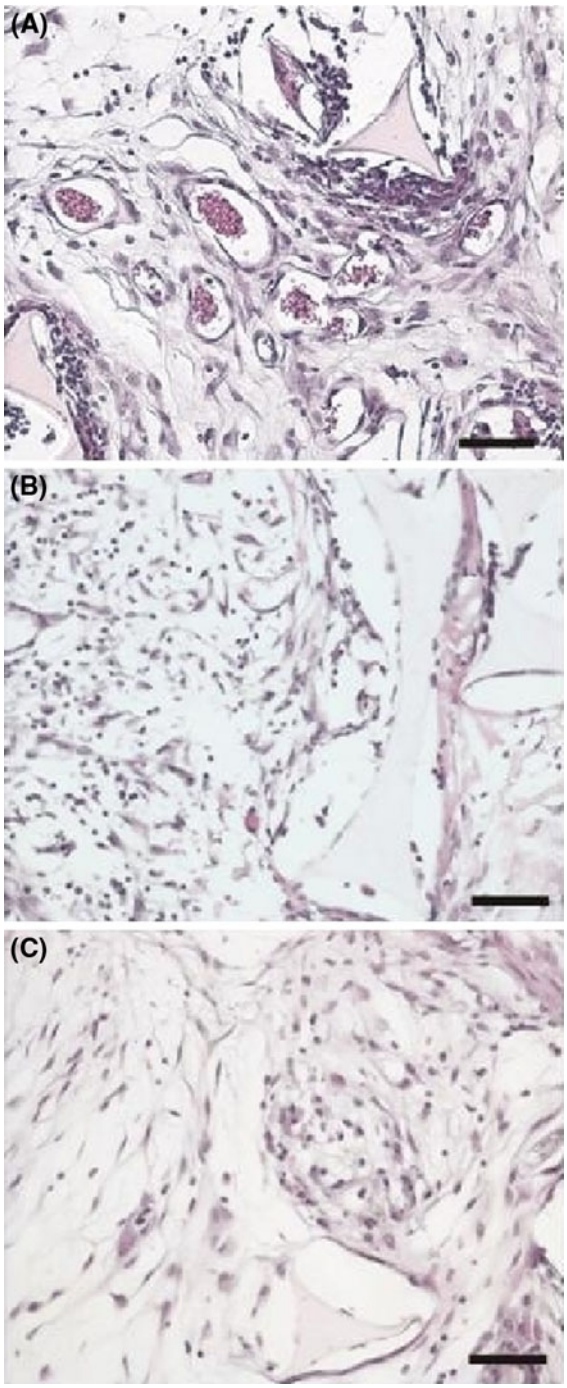

Fig. 4 Representative histological sections (5 μm, stained with H&E) of fibro-vascular tissue induced in subcutaneous sponge implants in Swiss mice at day 9 post implantation. The newly formed tissue in control implants (**a**) densely vascularized infiltrate with inflammatory cells, spindle-shaped fibroblasts. In lapachol- (**b**) and complex- (**c**) treated groups the number of vessels and inflammatory infiltrate are much lesser compared with the control group. Bar: 100 μm

leukocytes and microvessels. In lapachol- and complex-treated groups vascularization and inflammatory cell infiltrate were decreased.

Conclusions

Vascularization and inflammatory cell infiltrate were decreased in lapachol- and complex-treated groups. When i.p. administered lapachol and $[Bi(Lp)_2]Cl$ statistically reduced the hemoglobin content in the implants suggesting that reduction of neo-vascularization is caused by free lapachol in both experimental groups. On the other hand, in the *per os* treatment only complex $[Bi(Lp)_2]Cl$ statistically decreased the hemoglobin content in the implants. Similarly, NAG activity in the implants was statistically decreased by lapachol and $[Bi(Lp)_2]Cl$ in the i.p. treatment, while in the *per os* treatment inhibition was observed only by the bismuth(III) complex. In both i.p. and *per os* treatments, MPO activity, did not decrease in the implants suggesting that the studied compounds are unable to reduce the number of neutrophils in the assayed conditions.

Our results indicate that lapachol and complex $[Bi(Lp)_2]Cl$ present anti-angiogenic and anti-inflammatory properties on the fibro-vascular tissue induced by the synthetic matrix. The effects of $[Bi(Lp)_2]Cl$ are attributed to the lapachol ligand. However, coordination to bismuth(III) could be an interesting strategy for improvement of lapachol's therapeutic effects. In the employed model, the sponge induces an inflammatory angiogenic response that reproduces many features of the healing occurring after mechanical and natural injuries such as balloon angioplasty, atherosclerosis, inflamed synovium and surgical wounds (Rocha et al. 2006). Hence, lapachol and its bismuth(III) complex $[Bi(Lp)_2]Cl$ may present potential as drug candidates to be used to modulate the inflammatory and angiogenic components of a number of pathological conditions.

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