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Research Paper

Angiogenic and Cell Proliferating Action of the Natural Diarylnonanoids, Malabaricone B and Malabaricone C during Healing of Indomethacin-induced Gastric Ulceration

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Purpose. To evaluate the plant phenolics, malabaricone B (mal B) and malabaricone C (mal C) in healing stomach ulcer by modulating angiogenesis.
Materials and Methods. Male Swiss albino mice, ulcerated with indomethacin (18 mg/kg, p. o., single dose) were treated up to 7 days with different doses of mal B or mal C. The healing capacities of the drugs and their effects on the angiogenic parameters were assessed.
Results. Maximum ulceration, observed on the 3rd day after indomethacin administration was effectively healed by mal B and mal C (each 10 mg/kg, p. o.×3 days), the latter showing equivalent potency (~78% $p<0.001$) as that of Omez (3 mg/kg, p. o.×3 days) and misoprostol (10 μg/kg, p. o.×3 days). Compared to the untreated mice, those treated with mal B or mal C respectively for 3 days increased the mucosal EGF level (139 and 178%, $p<0.001$), the serum VEGF level (56%, $p<0.01$ and 95%, $p<0.001$) and microvessels formation (37%, $p<0.05$ and 62%, $p<0.01$), while reducing the serum endostatin level (37%, $p<0.05$ and 61%, $p<0.01$). The relative healing capacities of mal B and mal C correlated well with their respective abilities to modulate the angiogenic factors. The healing by Omez and misoprostol was not due to improved angiogenesis.
Conclusions. The drugs, mal B and mal C could effectively heal indomethacin-induced stomach ulceration in mice by promoting angiogenesis.

KEY WORDS: angiogenesis; EGF; endostatin; gastrointestinal toxicology; malabaricone; VEGF.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are associated with an increased prevalence of gastroduodenal ulceration (1) and their continued use in the presence of ulceration results in delayed healing (2,3). Consequently, prevention of NSAID-mediated gastrointestinal disorder continues to be of concern for both clinical practitioner and researchers. Several mechanisms including mucosal blood flow (4), cellular proliferation and migration at the ulcer edge, and maturation of granulation tissue at the ulcer base (5) contribute to ulcer healing. Angiogenesis, the formation of new blood vessels, is an important component of granulation tissue maturation and plays a crucial part in wound healing (6). Hence, drugs that can modulate the angiogenic response during acute inflammatory insult of the NSAIDs are potential targets for therapeutic research. In spite of their efficacy in managing the NSAID induced gastric ulceration, the currently available synthetic anti-ulcer drugs confer side effects, and are expensive especially for the rural population. Development of suitable formulations from dietary sources might provide antiulcer medications with less or no toxicity, and affordability.

The fruit rind of the plant *Myristica malabarica* (Myristicaceae) (popularly known as rampatri, Bombay mace, or false nutmeg) is used as an exotic spice in various Indian cuisines. Although not substantiated adequately, it is credited with hepatoprotective, anticarcinogenic, and antithrombotic properties, and is found as a constituent in many Ayurvedic preparations such as pasupasi. Very recently, we have found (7) that two of its constituent diarylnonanoids, malabaricone B (mal B) and malabaricone C (mal C), especially the latter possess better *in vitro* antioxidant activity than curcumin. The chemical structures of the malabaricones are shown in Fig. 1. Given that many anti-ulcer drugs exert their action *via* antioxidative activity (8), we were interested to study the healing property of mal B and mal C against indomethacin-induced acute gastric ulceration of mice *vis-à-vis* that of the commercial drugs, omeprazole (Omez) and misoprostol. Another objective of the investigation was to correlate the ulcer healing capacity of the test samples with their ability to

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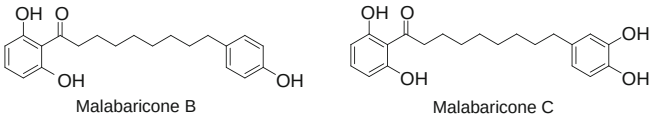

Fig. 1. Chemical structures of malabaricone B and malabaricone C.

modulate different angiogenic factors and influence the vascularity of granulation tissue in gastric ulcers of mice.

MATERIALS AND METHODS

Chemicals and Reagents

The malabaricones (designated as mal B and mal C respectively) were isolated from the methanol extract of dried fruit rind of *M. malabarica* as reported earlier (7). Indomethacin, bovine serum albumin (BSA), omeprazole (Omez), misoprostol, 3,3′-diaminobenzidine (DAB), rabbit anti-mouse EGF and Trizma base were procured from Sigma, St. Louis, USA. The vascular endothelial growth factor (VEGF) ELISA kit and peroxidase conjugated goat anti-rabbit IgG were from EMD Biosciences, Sandiego, USA while endostatin and von Willebrand Factor (rabbit anti-human) were from Chemicon, Temecula, USA. Other reagents used were hydrogen peroxide (35%, Lancaster, Morecambe, UK), disodium hydrogen phosphate and sodium dihydrogen phosphate from BDH, Poole Dorset, U.K, haematoxylin (monohydrate) and eosin yellowish (both from Merck, Mumbai, India), potash alum (S.D. Fine Chem., Mumbai, India), and horse and goat sera (Banaglore Genie, Banaglore, India).

Preparation of the Drugs

The drugs were prepared from mal B, mal C, Omez and misoprostol as aqueous suspensions in 2% gum acacia as the vehicle, and administered to the mice orally.

Experimental Protocol for Ulceration and Healing

The mice were bred at Dr. B. C. Roy Post Graduate Institute of Basic Medical Sciences, Kolkata, India and BARC Laboratory Animal House Facility, Mumbai, India. These were procured after obtaining clearance from the respective Animal Ethics Committees of the two centres and were handled following International Animal Ethics Committee Guidelines. Male Swiss albino mice (25–30 g) were reared on a balanced laboratory diet as per NIN, Hyderabad, India and given tap water ad libitum. They were kept at 20±2 °C, 65–70% humidity, and day/night cycle (12:12 h). At the beginning of each experiment, all animals were identified by typical notches in the ear and limbs and then randomized, to carry out the experiments in a blinded fashion. The animals were deprived of food but had free access to tap water 24 h before ulcer induction.

Ulceration in the mice was induced by administering a single dose of indomethacin (18 mg/kg, p. o.) dissolved in distilled water and suspended in 2% gum acacia. Our studies with 5, 10, 15, 18, 20, 25 and 30 mg/kg, p. o. of indomethacin revealed that the lowest doses (5 and 10 mg/kg) provided minor ulceration after 6 h of its administration, while the higher doses (25 and 30 mg/kg) led to mortality. The chosen dose (18 mg/kg) produced optimal ulceration, with inflammation and mucosal insult, without causing any mortality to mice.

Standardization of Doses of Mal B and Mal C

For this, mice were given mal B or mal C (2, 5, 10, 15 and 20 mg/kg) as a single dose per day up to seven days, starting from 6 h of indomethacin administration. Five mice were taken in each treatment regime and each experiment was repeated three times. The mice were sacrificed on the respective days 4 h after the administration of the drugs. The extent of healing was assessed from the macroscopic damage scores (MDS) of the untreated and treated ulcerated mice, measured on first, third, fifth and seventh days and the treatment regime (dose and time) of the drugs was optimized. During these experiments, the normal and ulcerated control mice of the respective days of ulceration were given the vehicle oral dose of gum acacia in distilled water (0.2 ml per mice) only.

Assessment of Ulceration and Healing from MDS

The mice were sacrificed after an overdose with thiopental. The stomach from the normal and treated groups were removed rapidly, opened along the greater curvature, and thoroughly rinsed with normal saline. The ulcerated gastric mucosal areas were visualized using a transparent sheet and a dissecting microscope. The MDS was assessed (9) by grading the gastric injury on a 0–4 scale, based on the severity of hyperemia and hemorrhagic erosions: 0–almost normal mucosa, 0.5–hyperemia, 1–one or two lesions, 2–severe lesions, 3–very severe lesions, 4–mucosa full of lesions. (lesions–hemorrhagic erosions, hyperemia–vascular congestions). The experiments were performed by two investigators blinded to the groups and treatment of animals.

Studies on the Histopathological and Biochemical Parameters

Our MDS results revealed that maximum ulceration in the untreated mice as well as best healing with the drug-treated mice were evident on the third day after indomethacin administration. Hence, we assessed the histopathological, and biochemical parameters under the optimized treatment regime [mal B and mal C (each 10 mg/kg), Omez (3 mg/kg) and misoprostol (10 μg/kg)] up to the 3rd day of ulceration only. The dose of Omez, which is also the recommended therapeutic dose for humans, was decided based on the results of our own studies (10). The dose of misoprostol was optimized by carrying out separate experiments with 2–15 μg/kg of the drug (data not shown). For the histopathological and biochemical assays, the following 11 groups of mice were selected from those used for the MDS assay: group I—normal mice; group II—ulcerated mice and sacrificed after 10 h (considered as 1 day); group III—ulcerated mice, and sacrificed after 3 days; groups IV–VII—ulcerated mice, treated with mal B, mal C, Omez and misoprostol respectively for 1 day, and sacrificed 4 h after administration of the drugs; groups VIII–XI—ulcerated mice, treated with mal B, mal C, Omez and misoprostol respectively for three days, and sacrificed 4 h after administration of the last dose of the drugs.

Histopathological Studies of Stomach Tissues

For the histological studies, the ulcerated portions of the stomach were sectioned after fixing in 10% formol saline solution. After 24 h of fixation followed by embedding in a paraffin block, it was cut into sections of 5 micron onto a glass slide, stained with haematoxylin–eosin and examined under a light microscope.

Quantification of Epidermal Growth Factor (EGF) Expression

For immunostaining EGF, the paraffin-embedded sections were processed following a reported procedure (11), with slight modification. Briefly, the stomach specimens were fixed in neutral-buffered formalin within 30 min of harvesting. After deparaffinization in xylene, the sections were treated with a graded series of alcohol and subsequently rehydrated in PBS at pH 7.5. Following blocking of the endogenous peroxidase activity with 3% hydrogen peroxide in PBS, samples were exposed to protein blockers (5% normal horse serum, 1% normal goat serum in PBS) and incubated overnight at 4°C with primary antibody at the appropriate dilution. In control sections, only PBS was added omitting the antibodies. After incubation for 1 h at room temperature with peroxidase conjugated goat anti-rabbit IgG, a positive reaction was detected by exposure to DAB for 2 to 5 min. The slides were counterstained with Meyer's haematoxylin, and the intensities of the immunolocalized areas were quantified using Biovis MV500 software. Five areas from each section were scanned and the integrated optical density (IOD) in each area was calculated. The IOD of the negative control was subtracted from the IOD of each experimental section for each animal in all the groups.

Assay of VEGF and Endostatin

The serum VEGF and endostatin were measured using the blood samples drawn from the descending aorta, with commercially available ELISA kits following manufacturer's instruction.

Quantification of Angiogenesis by von Willebrand Factor VIII (vWF VIII)

The number of microvessels in the ulcer was assessed from vWF, following a reported immunohistochemical procedure (12) with slight modifications. Briefly, following digestion of the tissue section with 0.1% trypsin, endogenous peroxidase activity as well as nonspecific protein binding sites were blocked. The sections were incubated with the polyclonal rabbit antihuman Factor VIII-related antigen for 2 h at room temperature and the peroxidase method was used to assay the formation of microvessels. Any positive-staining endothelial cells or endothelial cell clusters that were clearly separated from adjacent microvessels were considered an angiogenic microvessel. The vascular areas immediately adjacent to the normal tissue of the stomach served as internal quality controls. The microvessels (under ×400 magnification) on coded slides in five randomly selected microscopic fields of mucosal erosions were counted, and the data were averaged.

Acute Toxicity Assay of Mal B and Mal C on Mice

The acute toxicity of mal B and mal C on mice was studied by oral gavage of the drugs (each 500 mg/kg) and observing the animals for one month. The experiments were carried out with 15 mice and repeating the experiment twice. At the end of the observation period, the animals were sacrificed and the histology of the liver and kidney was assessed. For renal and liver function tests, animals were bled from the retro-orbital complex, and the serum of each mouse was analyzed for urea, creatinine, SGPT, and SGOT with an autoanalyser (Randox daytona, UK) using the respective kits.

Statistical Analysis

The data are presented as mean±S.E.M. Parametric data which includes all the biochemical parameters were analyzed using a paired 't' test for the paired data or one way analysis of variance (ANOVA) followed by a Dunnet multiple comparisons post test. Nonparametric data (macroscopic scoring) were analyzed using Kruskal–Wallis test (nonparametric ANOVA) followed by a Dunn's multiple comparisons post test. In addition to all the tests, Bonferroni correction was also carried out for knowing the simultaneous statistical inference among the groups under investigations. The IC_{50} values of the malabaricones were estimated using the Probit analysis and the significance level of the analyses was also investigated by the chi-square test. A probability value of $p<0.05$ was considered significant.

RESULTS

Standardization of Treatment Regime with Mal B and Mal C for Gastric Ulcer Healing

The time course of the extent of macroscopic damage due to gastric ulceration and its prevention by different doses of mal B and mal C are shown in Fig. 2a and b. The mice receiving vehicle only showed no lesions in the gastric mucosa. On third day of ulceration, the MDS value reached maximum increasing by ~107% compared to that on day one ($p<0.001$). The autohealing was prominent on the seventh day after ulceration, when the MDS value was reduced by 44.1% compared to that on day 1 ($p<0.01$).

Both mal B and mal C, at all the chosen doses showed maximum ulcer healing on the third day of ulceration, and the effect was dose-dependent. Compared to the respective ulcerated controls, treatment with mal B (10 and 20 mg/kg) for one and 3 days reduced the MDS by 17.8–24.3% ($p<0.05$) and 61.9–69.8% ($p<0.001$) respectively. The effects of mal B (10–20 mg/kg) were significantly better ($p<0.05$) than that at its lower dose (5 mg/kg).

In contrast, mal C showed much better results than mal B at any given dose and treatment period. For example, compared to the ulcerated untreated mice, the mice receiving mal C (10–20 mg/kg) showed a significant ($p<0.01$) reduction in the MDS even on the day of ulcer induction. Prolonging the treatment for three days reduced the MDS by 78.1–85.7% ($p<0.001$), compared to the group III mice. The results of the higher doses (10–20 mg/kg) of mal C were similar, and significantly different ($p<0.01$) from those receiving mal C

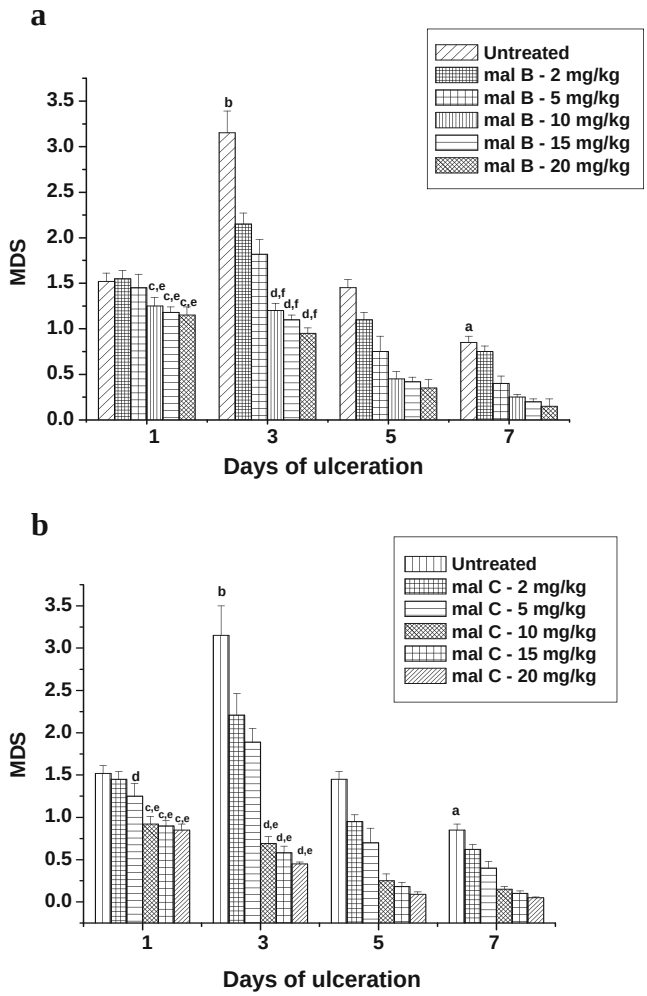

Fig. 2. a Comparative dose-dependent healing capacity of mal B against indomethacin-induced stomach ulceration in mice. Stomach ulceration in mice was induced by oral administration of indomethacin (18 mg/kg). Different doses of mal B were used for the experiments. The ulcer indices in terms of the macroscopic damage scores (*MDS*) were measured on different days after indomethacin administration and the values are mean±SEM (n=15). $^{a}p<0.01$; $^{b}p<0.001$ compared to normal mice; $^{c}p<0.05$, $^{d}p<0.001$ compared to respective ulcerated mice; $^{e}p<0.05$ compared to mice treated with 5 mg/kg of mal B. **b** Comparative dose-dependent healing capacity of mal C against indomethacin-induced stomach ulceration in mice. Stomach ulceration in mice was induced by oral administration of indomethacin (18 mg/kg). Different doses of mal C were used for the experiments. The ulcer indices in terms of the macroscopic damage scores (*MDS*) were measured on different days after indomethacin administration and the values are mean±SEM (n=15). $^{a}p<0.01$; $^{b}p<0.001$ compared to normal mice; $^{c}p<0.01$, $^{d}p<0.001$ compared to respective ulcerated mice; $^{e}p<0.05$ compared to mice treated with 5 mg/kg of mal C.

(5 mg/kg). In comparison, treatment with Omez (3 mg/kg) and misoprostol (10 μg/kg) for three days reduced the MDS by 76.3 and 78.8% respectively, compared to that in the untreated mice ($p<0.001$).

For the untreated mice, peak ulceration (maximum MDS) was observed on the third day of indomethacin administration. Hence, this time point was selected to find out the IC_{50} values of mal B and mal C. Considering the MDS values of the third day ulcerated untreated mice as 100%, the IC_{50} values of mal B and mal C were found to be 8.61±0.65 and 6.01±0.42 mg/kg respectively, which were significantly different ($p<0.05$; Fig. 3).

Effect of the Drugs on Biochemical Parameters

Overall, treatment with both mal B and mal C (10 mg/kg) for 3 days after ulcer induction provided optimal ulcer healing. Hence, only those mice receiving mal B, mal C (each 10 mg/kg), Omez (3 mg/kg) or misoprostol (10 μg/kg) up to 3 days were selected for assessing the angiogenic parameters. The comparative results of the untreated and treated groups of mice are summarized in Tables 1 and 2.

Macroscopic and Histological Assessment

At 6 h post indomethacin administration, macroscopic evaluation was performed on the basis of severity of hyperemia (vascular congestions) and hemorrhagic erosions. On the day of ulcer induction itself, hemorrhagic lesions covering the total glandular area of the stomach was evident with the untreated mice. However, mucosal hyperemia along with hemorrhage reached maximum on the third day of ulceration along with the disruption of the gel-like mucin cover in the untreated group. Administration of the drugs had immediate effect, reducing the glandular hemorrhagic lesions substantially. Prolonging the treatment for three days reduced the mucosal congestion and restored the hydrophobic mucous layer to near normalcy.

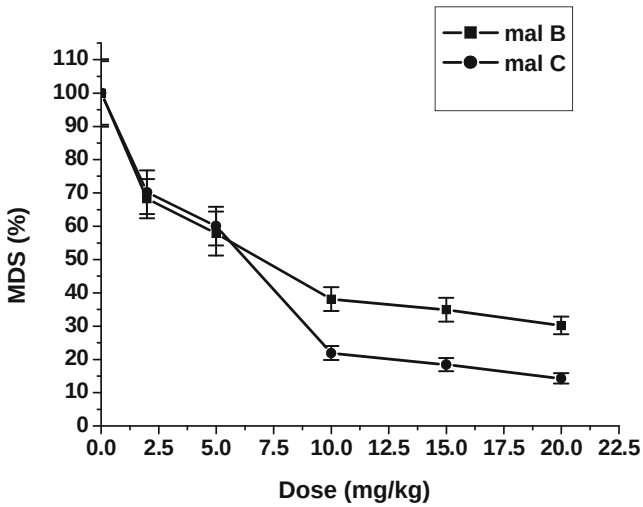

Fig. 3. Concentration-dependent healing capacities of mal B and mal C on the third day after indomethacin-induced stomach ulceration in mice as revealed from the macroscopic damage scores (MDS). Stomach ulceration in mice was induced by oral administration of indomethacin (18 mg/kg). Different doses of mal B and mal C were used for the experiments. The MDS values were measured 4 h after the last dose of the respective drugs, and normalized considering the MDS value of the third day ulcerated untreated mice as 100. The values are mean±SEM (n=15). The IC_{50} values of the test samples were determined by Probit analysis of the data and were found to be significantly different ($p<0.05$).

Table 1. The Effect of Indomethacin-Mediated Stomach Ulceration of Mice on the Levels of Serum VEGF and Endostatin, and Tissue EGF and vWF on the First Day of Ulceration and their Modulation by Mal B, Mal C, Omez and Misoprostol[a]

Parameters	Group I Normal control	Group II Ulcerated control	Group IV mal B-treated	Group V mal C-treated	Group VI Omez-treated	Group VII Misoprostol-treated
EGF (integrated O.D.×10^3)	2.31±0.21	6.24±0.55*	12.95±1.09***, ****, [b]	14.14±1.35***, ****, [b]	6.32±0.46	7.68±0.62**
VEGF (ng/ml)	10.09±0.97	9.05±0.52	8.89±0.89	8.66±0.77	8.19±0.69	9.39±0.86
Endostatin (ng/ml)	2.42±0.26	15.21±1.84*	15.61±1.58	14.07±1.12	15.95±1.88	14.39±1.54
vWF (microvessels/field)	36.14±3.58	35.58±3.58	32.54±2.8	36.85±2.65	33.85±2.88	36.01±3.45

[a] Stomach ulceration in mice was induced by oral administration of indomethacin (18 mg/kg). Mal B, mal C (each 10 mg/kg × 1 day), Omez (3 mg/kg × 1 day) and misoprostol (10 μg/kg × 1 day) were used as the drugs. The assays were carried out 4 h after the drug administration. The values are mean±SEM (n=15).
*p<0.001 compared to normal mice
**p<0.05 compared to ulcerated untreated mice
***p<0.001 compared to ulcerated untreated mice
****p<0.01 compared to misoprostol-treated mice
[b] p<0.001 compared to Omez-treated mice.

Table 2. The Effect of Indomethacin-Mediated Stomach Ulceration of Mice on the Levels of Serum VEGF and Endostatin, and Tissue EGF and vWF on the Third Day of Ulceration and their Modulation by Mal B, Mal C, Omez and Misoprostol[a]

Parameters	Group I Normal control	Group III Ulcerated control	Group VIII mal B-treated	Group IX mal C-treated	Group X Omez-treated	Group XI Misoprostol-treated
EGF (integrated O.D.×10^3)	2.30±0.22	8.20±0.89***	19.59±1.87[b,d]	22.81±1.99[b,d]	9.95±0.98****	11.41±1.61[a]
VEGF (ng/ml)	11.78±0.75	6.47±0.96**	10.12±1.62[a,d]	12.64±1.28[b,e]	7.15±0.95	7.90±0.84****
Endostatin (ng/ml)	2.64±0.35	25.15±2.15***	15.87±0.69****, [c]	9.71±0.93[a,d]	21.73±2.55	21.78±1.54
vWF (microvessels/field)	37.12±2.95	25.28±2.54*	34.54±3.42****, [c]	40.85±3.95[a,d]	26.65±2.54	29.54±2.75****

[a] Stomach ulceration in mice was induced by oral administration of indomethacin (18 mg/kg). Mal B, mal C (each 10 mg/kg×1 day), Omez (3 mg/kg×1 day) and misoprostol (10 μg/kg×1 day) were used as the drugs. The assays were carried out 4 h after the last dose of the drugs. The values are mean±SEM (n=15).
*p<0.05 compared to normal mice
**p<0.01 compared to normal mice
***p<0.001 compared to normal mice
****p<0.05 compared to ulcerated untreated mice
[b] p<0.01 compared to ulcerated untreated mice
[c] p<0.001 compared to ulcerated untreated mice
[d] p<0.05 compared to Omez and misoprostol-treated mice
[e] p<0.01 compared to Omez and misoprostol-treated mice
[f] p<0.001 compared to Omez and misoprostol-treated mice

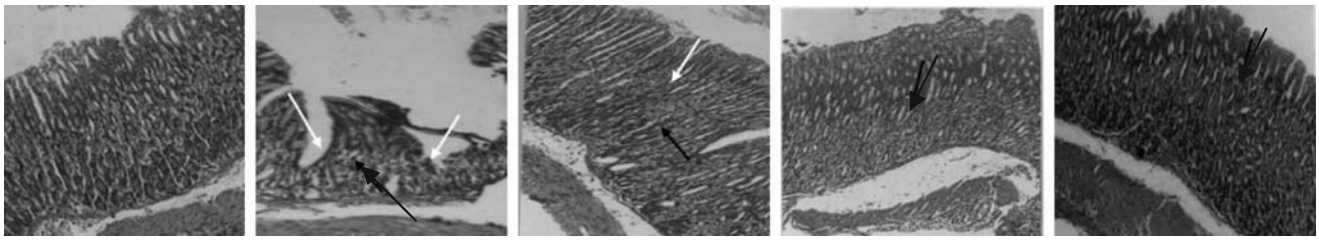

Fig. 4. Histological assessment of acute gastric mucosal injury induced by indomethacin (18 mg/kg) in mice and its prevention by mal B (10 mg/kg), mal C (10 mg/kg) and Omez (3 mg/kg). Section of mice stomachs obtained from **a** normal control mice on day 3; **b** untreated control mice on the third day of ulceration; **c–e** ulcerated mice treated with mal B, mal C and Omez for 3 days. *Black* and *white arrows* indicate inflammatory cells and mucosal damage, respectively.

Consistent with macroscopic observations, the histological examination of the untreated ulcerated mice stomach also revealed immediate (first day) superficial damage to the glandular portion. Subsequently, exfoliation of gastric epithelial cells, disruption of mucosal layer and strong infiltration by inflammatory cells were observed at peak ulceration. Treatment with the drugs showed re-epithelialization and progressive regeneration of the mucosal architecture starting from the first day. Treatment for three days helped in the proliferation of the gastric epithelial cells which migrated over and into the ulcer crater forming a region with maximal repair activity (Fig. 4a–e). Formation of microvessels was also apparent. Among the drugs, mal C, Omez and misoprostol promoted much faster ulcer healing than mal B.

Effect of the Drugs on Mucosal EGF of the Ulcerated Mice

Immunohistochemistry of the stomach tissue of mice (Fig. 5) showed that administration of the drugs led to an increased expression of EGF in mucosa of the ulcer margin. EGF was found to be immunolocalized in proliferative zone cells and in some parietal cells in the gastric oxyntic mucosa.

Quantification of the immunopositive areas revealed increased EGF expression by 2.7 ($p<0.001$) and 3.6 fold ($p<0.001$) respectively on the first and third days of ulceration in the untreated mice compared to that in normal mice. Treatment with mal B and mal C for 1 day increased the EGF expression by 107.6 and 126.6% respectively compared to that of the

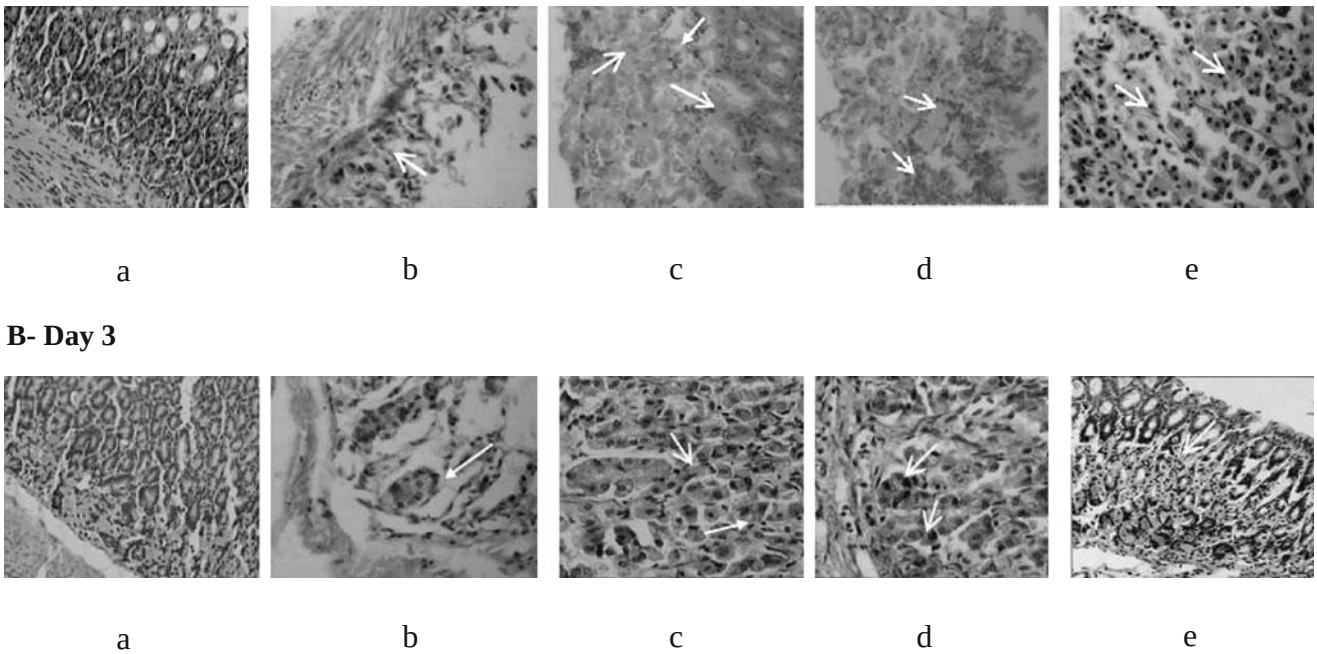

Fig. 5. Immunohistochemical staining of EGF expression in indomethacin administered acute gastric ulcers of mice and its modulation by mal B (10 mg/kg), mal C (10 mg/kg) and Omez (3 mg/kg). **A** EGF expression on day 1; **B** EGF expression on day 3. *a* normal control mice; *b* ulcerated untreated mice, *c–e* ulcerated mice treated with mal B, mal C and Omez. EGF immunostaining was carried out using the peroxidase conjugate. Original magnification ×400.

untreated mice ($p<0.001$). Prolonging the treatment for 3 days with mal B and mal C increased the EGF expression by 138.9 and 178.2% respectively, compared to the group III mice ($p<0.001$). Omez increased the EGF expression by 21.3% ($p<0.05$), without showing any immediate effect. However, misoprostol augmented it by 23.0% ($p<0.05$) and 39.1% ($p<0.01$) respectively compared to those of the group II and III mice.

Effect of the Drugs on the Serum VEGF and Endostatin Levels of the Ulcerated Mice

Indomethacin administration reduced the serum VEGF level by 45.1% on the third day of ulceration, compared to that normal mice ($p<0.01$). Compared to the untreated mice, treatment with mal B, mal C and misoprostol for 3 days increased the serum VEGF level by 56.4% ($p<0.01$), 95.4% ($p<0.001$) and 22.1% ($p<0.05$) respectively. The effects of mal B ($p<0.01$) and mal C ($p<0.001$) were significantly better than those of Omez and misoprostol. Omez did not alter it notably. However, our separate experiments carried out with higher doses (5, 10 and 15 mg/kg × 3 days) of Omez revealed that it could increase the VEGF level by ~20.0% compared to that of the group III mice ($p<0.05$) only at ≥10 mg/kg.

Indomethacin administration caused a marked upregulation of the serum endostatin level by 6.3 and 9.5 fold on the first and third days ulceration, compared to that of the normal mice ($p<0.001$). Treatment with mal B and mal C for three days reduced the level of serum endostatin by 36.9% ($p<0.05$), and 61.4% ($p<0.01$) respectively compared to the group III mice. In contrast, both Omez (3–10 mg/kg) and misoprostol (10 μg/kg) reduced the serum endostatin level by ~13.5% only, which was significantly less than that of mal B ($p<0.05$) and mal C ($p<0.01$). Even at higher doses, misoprostol did not alter the parameter significantly (data not shown).

Effect of the Drugs on vWF VIII of the Ulcerated Mice

The number of microvessels in the ulcerated mice was reduced by 31.9% than that in normal mice ($p<0.05$), without being affected on the day of ulcer induction. Compared to the group III mice, treatment with mal B and mal C for 3 days increased the mucosal microvessels by 36.6% ($p<0.05$) and 61.6% ($p<0.01$) respectively. Treatment with misoprostol for 3 days significantly enhanced the parameter (16.9%) compared to the ulcerated untreated animals ($p<0.05$). Omez (3 mg/kg× 3 days) was ineffective, but at a dose of 10 mg/kg, it increased the mucosal microvessels by 15.6% ($p<0.05$), compared to that of the group III mice.

Studies on Acute Toxicity of Mal B and Mal C in Mice

The possible toxic effect of mal B and mal C (each 500 mg/kg) on mice revealed no observable physical sign change, with normal food and water intake as well as stool during the experimental period. Normal hepatic microarchitecture, laminar arrangement of hepatocyte, central vein, portal triad, and biliary canaliculi without any inflammatory infiltrate or necrosis were observed in the mice livers receiving the drugs. Likewise, normal renal micro-architecture with well-differentiated cortex and medulla and without hemorrhage, or inflammation was found in the kidneys.

DISCUSSION

Clinical and experimental data indicate that, besides inducing gastric ulceration, the NSAIDs also delay the healing of gastroduodenal ulcers by interfering with the action of growth factors, decreasing epithelial cell proliferation in the ulcer margin, decreasing angiogenesis in the ulcer bed, and slowing maturation of the granulation tissue (13). Angiogenesis, requiring the concerted interaction of a variety of cellular systems is a pivotal process in all types of wound healing, including the healing of gastric ulcers (14). It is regulated by proangiogenic factors such as VEGF, fibroblast growth factor, and EGF, as well as antiangiogenic factors (such as endostatin). An imbalance in the production of antiangiogenic *versus* proangiogenic factors could result in impaired angiogenesis and ulcer healing (15). It is possible that the differential efficacy of some of the ulcer-healing drugs could be attributed to their divergent effects on angiogenesis.

In the present study, we studied the possible healing effects of mal B and mal C against indomethacin-induced acute gastric lesions in mice, and rationalized their potency with their capacity in augmenting the growth of new blood vessels and modulating different angiogenic factors. For this, we focused on EGF, VEGF, and endostatin, and also analyzed the microvessel formation.

Both proton pump inhibitors (PPI) and prostaglandin (PG) preparations are believed to prevent NSAID-induced gastric ulceration and circumvent the delayed healing. But it remains unclear which of these drugs is superior. Hence, we compared the efficacy of mal B and mal C with that of Omez, a PPI, and misoprostol, a PGE_1 analogue. We choose Omez as one of the positive controls, since it is reported to have greater efficacy and tolerability in the management of NSAID-associated GI side effects including those in clinical conditions (16,17). On the other hand, the choice of misoprostol was obvious, considering its specific use against the NSAID-induced gastropathy. Apart from counteracting the inhibition of PG production and reducing the secretion of gastric acid, it also maintains the gastric mucosal barrier and mucosal blood flow (1,18).

Our macroscopic and histopathological examinations revealed that administration of indomethacin caused marked damage to the gastric mucosa with elongated hemorrhagic lesions in the glandular portion. The maximum ulcerative damage, observed on the third day after indomethacin administration, was, however, acute in nature as evident from natural recovery of the gastric tissues even without any treatment. However, the healing was only partial (~44%) even after seven days. In comparison, the mice treated with mal B, mal C, Omez and misoprostol showed significantly faster and better healing within three days. The effects of mal C, Omez and misoprostol were comparable, and much better than that of mal B. The healing observed on extending the treatment up to seven days with both mal B and mal C was only marginally better than that observed with the three-day treatment regime. However, a major part of this was due to natural healing.

The growth factor, EGF accelerates gastroduodenal ulcer healing by stimulating cell migration and proliferation in epithelial cell monolayers, tissue repair, increasing release of gastric mucin, and attenuating gastric acid secretion (19). EGF increases the MAP kinase activity by activating the EGF receptor, and also stimulates PG synthesis that keep the gastrointestinal cell loss/ renewal tightly regulated to prevent ulceration and hyperplasia (20,21). A marked reduction in the concentration of EGF has been reported in the gastric juice of both duodenal and gastric ulcer in a larger population of patients in the active stage (22). The role of the endogenous EGF in ulcer healing is also unequivocally confirmed (23).

Our result on the increase in EGF level of the ulcerated mice over that of normal control mice is consistent with the requirement of more EGF for ulcer healing. This was increased further by mal B and mal C, while misoprostol and Omez showed significantly less effect. The present finding with mal B and mal C appears promising given that drugs that can stimulate endogenous EGF level are attractive for treating gastric ulcer.

Of the many growth factors, VEGF promotes endothelial proliferation and migration, and accelerate ulcer healing (24). It promotes restoration of the connective tissue and microvessels (angiogenesis) in injured mucosa. Indomethacin inhibits ADP-induced platelet aggregation and release of the α-granule, which stores VEGF (25). Consequently, indomethacin treatment would reduce VEGF release. In contrast, endostatin, the most potent inhibitor of angiogenesis (26) acts *via* inhibition of endothelial cell growth and migration, apoptosis promotion, and antagonization of VEGF (27).

We also observed significantly reduced serum VEGF, and increased endostatin levels due to the indomethacin-induced ulceration. Three-day treatment with mal B and mal C reversed these changes simultaneously. In contrast, although misoprostol and omeprazole provided excellent healing, their effect in modulating VEGF and endostatin levels was marginal.

Gastric ulcer healing entails several distinct repair mechanisms. The epithelial cell proliferation and migration from the ulcer edge across the ulcer bed is accompanied by maturation of granulation tissue beneath the ulcer base. Within this tissue vascular endothelial cells form new capillaries to restore the microvasculature, while fibroblasts restore the lamina propria. The degree of neovascularisation, as assessed by using specific endothelial markers including vWF, CD31, and CD34 in experimental ulcer models correlates well with the extent and speed of ulcer healing. Among the markers, vWF acts as a cofactor for platelet binding to expose extracellular matrix in injured vessel walls. It is implicated in the angiogenic functions of VEGF, and is reported to increase endothelial cell adhesion, helping maintenance of endothelial integrity (28). The endothelial vWF secretion (29), which is also induced by VEGF is crucial for platelet adhesion to subendothelial collagen, and upregulation of tissue factor.

With regard to NSAID's interference with angiogenesis during gastric ulcer healing, our studies revealed that indomethacin administration led to a progressive reduction of the number of microvessels in granulation tissues. Maximum reduction of the number of microvessels was observed on the third day of ulceration, reflecting inhibition of angiogenesis and the associated delay in ulcer healing in mice. Treatment with mal B, mal C and misoprostol, but not Omez for three days increased the number of microvessels significantly compared to that in the untreated ulcerated mice. The superior result with mal C was consistent with its better ability to increase the proangiogenic factors (EGF, VEGF), and reduce the serum endostatin level, compared to mal B.

In contrast to the malabaricones, modulation of angiogenesis was not the major contributing factor in the ulcer healing by the low doses of Omez, as reported earlier (30). Possibly, at these doses, Omez acts *via* its anti-secretary (31) and antioxidant action (8). Interestingly, the ulcer healing by Omez (10 mg/kg×3 days) was associated with increased angiogenesis as revealed from the increase in VEGF and vWF levels. However, at ≥10 mg/kg, Omez might be promoting cell proliferation and angiogenesis as reported earlier (32).

Overall, besides causing ulceration, indomethacin also delayed ulcer healing. Its significant effect in modulating serum levels of endostatin and VEGF levels was consistent with impairment of ulcer healing. In normal mice, both these factors were detected in serum. Both the test drugs, mal B, and mal C could accelerate the healing of gastric ulcer within three days of treatment, compared to normal healing without any treatment. Between these, mal C was more potent, showing equivalent efficacy as that of the commercial drugs, Omez and misoprostol. However, while mal C enhanced the angiogenesis for the healing, the mode of action of Omez, at the lower doses was different. Both mal B and mal C were found to be non-toxic to mice even at a very high single dose of 500 mg/kg. All these results favored mal C as a most potent ulcer-healing drug for further evaluation.

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