New Gastroprotective Ferruginol Derivatives with Selective Cytotoxicity against Gastric Cancer Cells

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Abstract

The diterpene ferruginol has shown a strong protective effect in animal gastric ulcer models. In the present work, we report the gastroprotective effect and cytotoxicity of 16 new semisynthetic ester derivatives of ferruginol. The gastroprotective effect of these compounds was assessed with the HCl/EtOH-induced gastric lesions model in mice and the cytotoxicity was measured using MRC-5 fibroblasts, gastric adenocarcinoma (AGS) and liver hepatoma Hep G2 cells. The compounds were tested for a gastroprotective effect at a single oral dose of 20 mg/kg. The best gastroprotective effect was elicited by ferruginyl nicotinate (**13**), reducing the lesion index by 71%, while the derivatives ferruginyl chloroacetate (**2**), ferruginyl palmitate (**6**), ferruginyl oleate (**7**), ferruginyl 3,5-dinitrobenzoate (**11**), ferruginyl 3-methylbenzofuran-2-carbonyl ester (**12**), ferruginyl indoleacetate (**14**), ferruginyl indolebutyrate (**15**) and ferruginyl pthalate (**16**) reduced the lesions by 49–66%. The most promising compounds were **11**, **13** and **14**, presenting a gastroprotective effect higher or similar to that of ferruginol but with a high selectivity towards the tumor AGS cells. Among the three products, the most selective towards AGS cells was **14**, followed by **13**, and **11** (IC_{50} values of 12, 22 and 29 μM, respectively). The isobutyrate **4**, inactive as a gastroprotective agent, showed selective cytotoxicity against AGS and Hep G2 cells (IC_{50} values of 60 and 39.2 μM, respectively). The cytotoxicity of the above cited compounds towards fibroblasts was >1000 μM. Considering the aliphatic esters of ferruginol, the best gastroprotective activity was found in the C_{16} and C_{18} derivatives but tended to decrease with increasing aliphatic chain unsaturation. For short-chain esters, the gastroprotective effect could be observed when the chain contained a chlorine atom. For aromatic esters, the presence of nitro groups or a nitrogen atom in the aromatic ring enhanced the gastroprotective activity. The compounds with the best gastroprotective effect and the highest selectivity against tumor cells bear an amino group (indoleacetate and nicotinate) or nitro group (3,5-dinitrobenzoate).

Abbreviations

AGS cells:	human gastric adenocarcinoma cells
DMSO:	dimethyl sulphoxide
FBS:	fetal bovine serum
HEP G2 cells:	human liver hepatocellular carcinoma cells
MEM:	minimum essential Eagle's medium
MRC-5 cells:	human lung fibroblasts
NRU:	neutral red uptake

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Around 8–10% of the population living in industrialized countries are affected by gastric and duodenal ulcers. In 2002, stomach cancer and peptic ulcer diseases represented 1.5 and 0.5% of the total causes of death in the world, respectively [1]. Recently, it has been well established that chronic gastric ulcer might lead to the development of gastric and pancreatic cancers [2], [3].
Abietane diterpenes have been found to be a promising source of bioactive compounds [4]. Ferruginol is an abietane diterpene occurring in plants belonging to the families Podocarpaceae

[5], Cupressaceae [6], Lamiaceae [7], [8] and Verbenaceae [9], among others [10]. Ferruginol exhibits different biological activities including antioxidative [9], miticidal [11], antifungal and antibacterial [12], cardioactive [13], antiplasmodial [14] and antitumoral [15]. Recently, it was reported that ferruginol displays a gastroprotective effect in animal models of induced gastric lesions. Furthermore, the diterpene accelerates the gastric ulcer healing process after subchronic ulcer induction in animals [16]. These effects have been related with the capacity of ferruginol to increase the gastric prostaglandin content *in vitro*, a stimulating effect on cell proliferation and the antioxidant properties of the compound [16]. In spite of these valuable properties as a gastroprotective agent, ferruginol proved to be a cytotoxic compound with IC_{50} values ranging from 24 to 26 μM towards both human normal and tumor cells [16]. In order to obtain better activity/cytotoxicity ratios, Areche et al. [17] prepared 18 ferruginol derivatives by semisynthesis. Some of the semisynthetic compounds were more active as gastroprotective agents than the parent compound. In addition, some of them proved to be less cytotoxic than ferruginol against normal fibroblasts and gastric cancer cells [17]. Interestingly, the derivative 12-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopiranosyloxy)-abieta-8,11,13-triene, that proved to be as active as ferruginol in protecting the gastric mucosa, showed a selective cytotoxicity against gastric cancer cells (at least four times higher compared to normal fibroblasts) [17].

In an attempt to obtain compounds with better antiulcer activity and selective cytotoxicity, we prepared 16 new ferruginol derivatives **2 – 17**, ◉ **Fig. 1**. The gastroprotective effects as well as the cytotocity towards normal human fibroblasts, human gastric cancer cells and human hepatoma cells are presented in this report.

Materials and Methods

General procedure

Melting points were determined on a Köffler hot stage apparatus (Electrothermal 9100) and were uncorrected. Optical rotations were obtained for solutions in $CHCl_3$ (concentrations expressed in g/100 mL) on a Jasco DIP 370 polarimeter. IR spectra were recorded on a Nicolet Nexus FT-IR instrument. ^{1}H-NMR spectra were recorded at 400 MHz, and ^{13}C-NMR data were obtained at 100 MHz on a Bruker Avance spectrometer (and expressed in δ). Mass spectra were obtained on an MAT 95XP, Thermo Finnigan spectrometer using electron ionization and perfluorotributylamine (Fluorinert FC-43) (Sigma Chemical Co.) as a reference and are presented as *m/z* (rel. int. %). Silica gel 60 (Merck, 63 – 200 μm particle size) was used for column chromatography, precoated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) were used for TLC analysis. Gel permeation was performed on Sephadex LH-20 (Pharmacia). TLC spots were visualized by spraying the chromatograms with *p*-anisaldehyde-ethanol-acetic acid-H_2SO_4 (2 : 170 : 20 : 10 v/v) and heating at 110 °C. All reactions were carried out under an inert dry nitrogen atmosphere.

Preparation of derivatives

Ferruginol was isolated from the wood of *Podocarpus nubigena* Lind. and from the stem bark of *Prumnopitys andina* (Poepp. ex Endl.) de Laub. (Podocarpaceae) as described previously [17]. The plants were identified by Dr. Patricio Peñaillillo, Departamento de Botánica, Universidad de Talca, and voucher herbarium specimens (N° 3008 and 3009, respectively) were deposited at the Herbarium of the Universidad de Talca. For the preparation of derivatives **2 – 11** as well as **13 – 17**, 150 mg of ferruginol were used. The amount of 1,3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) employed in all the mentioned reactions was 140 mg and 13 mg, respectively. Esterification of ferruginol was performed using chloroacetic (111 mg), iodoacetic (127 mg), isobutyric (60 mg), lauric (137 mg), palmitic (175 mg), oleic (296 mg) and linoleic (319 mg) acids (Sigma Chemical Co.) with (DCC/DMAP) in CH_2Cl_2 as solvent for 24 h at room temperature [18] affording the corresponding esters in 86 %, 35 %, 89 %, 54 %, 67 %, 61 % and 63 % yields for compounds **2 – 8**, respectively. Treatment of ferruginol with salicylic (94 mg), phenylacetic (93 mg) and 3,5-dinitrobenzoic (145 mg) acids (Sigma Chemical Co.) using the same protocol as described above afforded compounds **9 – 11** in 32 %, 99 % and 10 % yields, respectively. Esterification of ferruginol (100 mg) using 3-methylbenzofuran-2-carbonyl chloride (204 mg) (Sigma Chemical Co.) in pyridine at 0 °C under an inert atmosphere afforded the ester **12** in 7 % yield. Treatment of ferruginol with nicotinic (84 mg), indoleacetic (110 mg), indolebutyric (139 mg), phthalic (57 mg) and succinic (81 mg) acids (Sigma Chemical Co.) and DCC/DMAP in CH_2Cl_2 gave the corresponding esters in 99 %, 73 %, 67 %, 20 % and 10 % yields, for compounds **13 – 17**, respectively. The purity of all the derivatives was over 98 % as assessed by ^{1}H-NMR spectroscopy.

Animals

Male Swiss albino mice from the Instituto de Salud Pública de Chile, weighing 30 ± 3 g were used. The animals were fed on certified Champion diet with free access to water under standard conditions of 12 h dark-light period, 50 % relative humidity and 22 °C room temperature.

HCl/ethanol-induced gastric lesions

Mice were randomly distributed into groups of seven or eight animals each and fasted for 24 h with free access to water prior to the experiment. For the comparison of the ferruginol derivatives, a single oral dose of 20 mg/kg was selected because in a previous experiment we had determined that the lesion index was reduced by about 50 % by ferruginol at 20 mg/kg [16], [17]. Compounds were suspended in a 12 % solution of the non-ionic detergent Tween 80 (Sigma Chemical Co.) at a concentration of 2 mg/mL. Fifty minutes after oral administration of the compounds, or the positive control lansoprazole [2-({[3-methyl-4-(2,2,2-trifluroethoxy)-2-pyridyl]methyl}sulfinyl) benzimidazole; Sigma Chemical Co.], > 98 % purity by HPLC (20 mg/kg), or the vehicle 12 % Tween 80 (10 mL/kg), all groups were orally treated with 0.2 mL of a solution containing 0.3 M HCl/60 % ethanol (HCl/EtOH) for gastric lesion induction. Animals were sacrificed 1 h after the administration of HCl/ethanol, and the stomachs were excised and inflated by injection of saline (1.0 mL). The ulcerated stomachs were fixed in 5 % formalin for 30 min and opened along the greater curvature. Gastric damage visible to the naked eye was observed in the gastric mucosa as elongated black-red lines, parallel to the long axis of the stomach similar to the HCl/EtOH-induced lesions in rats. The length (mm) of each lesion was measured, and the lesion index was expressed as the sum of the length of all lesions. The percentage of gastroprotective effect was calculated as follows:

% lesion reduction = (1 – lesion index of sample/lesion index of control) × 100

The protocols were approved by the Universidad de Talca Institutional Animal Care and Use Committee that follows the recommendations of the Canadian Council on Animal Care [19].

MRC-5 cell culture

Human normal lung fibroblasts MRC-5 (ATCC CCL-171) were grown as monolayers in minimum essential Eagle's medium (MEM), with Earle's salts, 2.0 mM L-glutamine (Sigma Chemical Co.) and 2.2 g/L sodium bicarbonate (Sigma Chemical Co.), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin in a humidified incubator with 5% CO_2 in air at 37 °C. Culture media, antibiotics and fetal bovine serum were obtained from Invitrogen Corp. Cell passage was maintained between 10 and 16. The medium was changed every 2 days.

AGS cell culture

Human gastric adenocarcinoma cells AGS (ATCC CRL-1739) were grown as monolayers in Ham F-12 medium containing 1.0 mM L-glutamine and 1.5 g/L sodium bicarbonate, supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin in a humidified incubator with 5% CO_2 in air at 37 °C. The cell passage was maintained between 42 and 48. The medium was changed every 2 days.

Hep G2 cell culture

Human liver hepatocellular carcinoma cells Hep G2 (ATCC HB-8065) were grown as monolayers in minimum essential Eagle's medium (MEM), with Earle's salts, 2.0 mM L-glutamine and 2.2 g/L sodium bicarbonate, supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin in a humidified incubator with 5% CO_2 in air at 37 °C. Cell passage was maintained between 79 and 82. The medium was changed every 2 days.

Cytotoxicity assay

Confluent cultures of MRC-5, AGS or Hep G2 cells were treated with medium containing the diterpene derivatives as well as with the reference compounds at concentrations of 0, 200, 400, 600, 800 and 1000 μM. The products were first dissolved in DMSO (Sigma Chemical Co.) and then in the corresponding culture medium supplemented with 2% FBS. The final content of the solvent DMSO in the test medium and controls was 1%. Cells were exposed for 24 h to the test medium with or without the compound (control). Stability of the compounds in the culture medium was confirmed by means of TLC. Cells treated with lansoprazole or vinblastine (Sigma Chemical Co.) ≥ 97% purity by TLC, were used as positive controls for cytotoxicity. Each concentration was tested in quadruplicate together with the control and repeated three times in separate experiments. At the end of the incubation, the neutral red uptake (NR) (Sigma Chemical Co.) assay was carried out [20]. To calculate the IC_{50} values (concentration that produces a 50% inhibitory effect on the evaluated parameter), the results were transformed to percentage of controls assuming that they represent the 100% of cell viability. The IC_{50} values were graphically obtained from the dose-response curves.

Statistical analysis

Results were expressed as the mean ± S.D. In all experiments, statistical differences between several treatments and their respective control were determined by one-way analysis of variance (ANOVA) and when the F value was significant, post hoc differences were determined by the Dunnett's multiple comparison tests. The level of significance was set at $P < 0.01$. All statistical analyses were performed using the software Statistica 5.1 (StatSoft, Inc.) and Statistical Package S-Plus 2000.

Compounds

Ferruginol (**1**): White powder; m. p. 50 – 53 °C; $[\alpha]_D^{20}$: + 38.0 (*c* 0.5, $CHCl_3$); HR-MS (EI): *m/z* = 286.2297 (calcd. for $C_{20}H_{30}O$: 286.2297). For NMR data see [21] and [17].

Ferruginyl chloroacetate (**2**): Colorless powder; m. p. 128 – 130 °C; $[\alpha]_D^{20}$: + 51.8 (*c* 0.06, $CHCl_3$); IR (film): ν_{max} = 2916, 2844, 1752, 1166, 1130 cm^{-1}; HR-MS (EI): *m/z* = 362.20119 (calcd. for $C_{22}H_{31}ClO_2$: 362.20126); EI-MS: *m/z* (rel. int. %) = 364 (20) ($C_{22}H_{31}{}^{37}ClO_2$), 362 (58) ($C_{22}H_{31}{}^{35}ClO_2$), 349 (38), 347 (100), 319 (6), 305 (12), 286 (23), 277 (31), 265 (73), 250 (34), 201 (22), 187 (27), 147 (38), 77 (30), 69 (98).

Ferruginyl iodoacetate (**3**): Colorless powder; m. p. 101 – 102 °C; $[\alpha]_D^{20}$: + 38.2 (*c* 0.06, $CHCl_3$); IR (film): ν_{max} = 2964, 2920, 1744, 1501, 1242, 1162, 1083 cm^{-1}; HR-MS (EI): *m/z* = 454.13408 (calcd. for $C_{22}H_{31}IO_2$: 454.13693); EI-MS: *m/z* (rel. int. %) = 454 (16), 439 (8), 356 (6), 327 (4), 286 (100), 271 (40), 229 (6), 201 (12), 175 (16), 147 (18), 69 (29).

Ferruginyl isobutyrate (**4**): Colorless crystals; m. p. 76 – 77 °C; $[\alpha]_D^{20}$: + 52.2 (*c* 0.09, $CHCl_3$); IR (film): ν_{max} = 2960, 2868, 1752, 1465, 1126, 772 cm^{-1}; HR-MS (EI): *m/z* = 356.27193 (calcd. for $C_{24}H_{36}O_2$: 356.27155); EI-MS: *m/z* (rel. int %) = 356 (11), 286 (100), 271 (34), 229 (3), 201 (8), 189 (9), 175 (9), 159 (4), 149 (3), 147 (9), 71 (15), 69 (10), 55 (5).

Ferruginyl laurate (**5**): Colorless oil; $[\alpha]_D^{20}$: + 33.9 (*c* 0.06, $CHCl_3$); IR (film): ν_{max} = 2916, 2852, 1756, 1465, 1166, 1142 cm^{-1}; HR-MS (EI): *m/z* = 468.39614 (calcd. for $C_{32}H_{52}O_2$: 468.39673); EI-MS: *m/z* (rel. int. %) = 468 (2), 286 (100), 2719 (22), 201 (6), 189 (7), 183 (8), 175 (7), 149 (8), 147 (7), 71 (5), 69 (14), 57 (15), 55 (14).

Ferruginyl palmitate (**6**): Oily solid; $[\alpha]_D^{20}$: + 36.2 (*c* 0.07, $CHCl_3$); IR (film): ν_{max} = 2924, 2848, 1760, 1166, 1134 cm^{-1}; HR-MS (EI): *m/z* = 524.45718 (calcd. for $C_{36}H_{60}O_2$: 524.45935); EI-MS: *m/z* (rel. int. %) = 524 (2), 427 (0.5), 286 (100), 271 (17), 239 (5), 201 (5), 189 (5), 175 (5), 149 (8), 69 (9), 57 (9).

Ferruginyl oleate (**7**): Colorless oil; $[\alpha]_D^{20}$: + 23.7 (*c* 0.08, $CHCl_3$); IR (film): ν_{max} = 2924, 2852, 1760, 1461, 1162 cm^{-1}; HR-MS (EI): *m/z* = 550.47177 (calcd. for $C_{38}H_{62}O_2$: 550.47498); EI-MS: *m/z* (rel. int. %) = 550 (1), 453 (0.25), 286 (100), 271 (20), 201 (7), 175 (9), 149 (10), 83 (11), 69 (31), 55 (36).

Ferruginyl linoleate (**8**): pale yellow oil; $[\alpha]_D^{20}$: + 30.4 (*c* 0.26, $CHCl_3$); IR (film), ν_{max} = 2932, 2848, 1756, 1461, 1162, 1138 cm^{-1}; HR-MS (EI): *m/z* = 548.45747 (calc. for $C_{38}H_{60}O_2$: 548.4593).

Ferruginyl salicylate (**9**): Colorless crystals; m. p. 101 – 102 °C; $[\alpha]_D^{20}$: + 58.1 (*c* 0.09, $CHCl_3$); IR (film): ν_{max} = 3410, 2964, 2850, 1684, 1298, 1154, 760 cm^{-1}; HR-MS (EI): *m/z* = 406.25049 (calcd. for $C_{27}H_{34}O_3$: 406.25079); EI-MS: *m/z* (rel. int. %) = : 406 (17), 286 (100), 271 (29), 245 (10), 189 (12), 147 (9), 121 (79), 93 (10), 69 (12), 65 (13).

Ferruginyl phenylacetate (**10**): Colorles crystals; m. p. 98 – 99 °C; $[\alpha]_D^{20}$: + 51.4 (*c* 0.07, $CHCl_3$); IR (film): ν_{max} = 2960, 2868, 1752, 1489, 1234, 1122 cm^{-1}; HR-MS (EI): *m/z* = 404.27005 (calcd. for $C_{28}H_{36}O_2$: 404.27155); EI-MS: *m/z* (rel. int. %) = 404 (6), 286 (85), 271 (28), 201 (9), 189 (10), 187 (7), 175 (10), 147 (14), 119 (6), 91 (100), 69 (14).

Ferruginyl 3,5-dinitrobenzoate (**11**): Pale yellow crystals; m. p. 190 – 192 °C; $[\alpha]_D^{20}$: + 51.9 (*c* 0.05, $CHCl_3$); IR (film): ν_{max} = 2928, 2888, 1732, 1549, 1346, 1166, 772 cm^{-1}; HR-MS (EI): *m/z*

= 480.22196 (calcd. for $C_{27}H_{32}N_2O_6$: 480.22601); EI-MS: *m/z* (rel. int. %) = 480 (38), 465 (78), 395 (33), 383 (75), 381 (13), 369 (40), 286 (19), 285 (18), 271 (13), 195 (100), 149 (43), 75 (31), 69 (99), 55 (23).

Ferruginyl 3-methylbenzofuran-2-carbonyl ester (**12**): Colorless crystals; m. p. 133 – 135 °C; $[\alpha]_D^{20}$: + 54.3 (*c* 0.04, $CHCl_3$); IR (film): ν_{max} = 2932, 2860, 1078, 1593, 1290, 1134, 1087, 752 cm^{-1}; HR-MS (EI): *m/z* = 444.26628 (calcd. for $C_{30}H_{36}O_3$: 444.26645); EI-MS: *m/z* (rel. int. %) = 444 (11), 429 (3), 285 (31), 159 (100), 149 (6), 103 (7), 85 (18), 83 (28), 69 (8), 55 (10).

Ferruginyl nicotinate (**13**): Colorless powder; m. p. 109 – 110 °C; $[\alpha]_D^{20}$: + 56.6 (*c* 0.05, $CHCl_3$); IR (film): ν_{max} = 2964, 2860, 1740, 1585, 1266, 1162, 1087, 756 cm^{-1}; HR-MS: *m/z* = 391.25168 (calcd. for $C_{26}H_{33}NO_2$: 391.25116); EI-MS: *m/z* (rel. int. %) = 391 (17), 376 (21), 334 (4), 308 (9), 294 (15), 285 (14), 280 (8), 147 (9), 106 (100.00), 78 (46), 69 (11), 51 (13).

Ferruginyl indoelacetate (**14**): Colorless crystals; m. p. 67 – 69 °C; $[\alpha]_D^{20}$: + 39.7 (*c* 0.06, $CHCl_3$); IR (film): ν_{max} = 3408, 2926, 2868, 1740, 1242, 1119 cm^{-1}; HR-MS (EI): *m/z* = 443.28300 (calcd. for $C_{30}H_{37}NO_2$: 443.28243); EI-MS: *m/z* (rel. int. %) = 443 (14), 286 (14), 271 (8), 157 (100), 130 (80).

Ferruginyl indolebutyrate (**15**): Colorless crystals; m. p. 56 – 58 °C, $[\alpha]_D^{20}$: + 40.4 (*c* 0.05, $CHCl_3$); IR (film): ν_{max} = 3414, 2972, 2928, 1744, 1453, 1138, 740 cm^{-1}; HR-MS (EI): *m/z* = 471.31136 (calcd. for $C_{32}H_{41}NO_2$: 471.31375); EI-MS: *m/z* (rel. int. %) = 471 (6), 286 (100), 271 (32), 186 (82), 130 (34).

Ferruginyl pthalate (**16**): Colorless crystals; m. p. 120 – 122 °C; $[\alpha]_D^{20}$: + 36.0 (*c* 0.05, $CHCl_3$); IR (film): ν_{max} = 2956, 2920, 1736, 1493, 1266, 1246, 1162, 1111, 756 cm^{-1}; HR-MS (EI): *m/z* = 702.46031 (calcd. for $C_{48}H_{62}O_4$: 702.46484); EI-MS: *m/z* (rel. int. %) = 702 (1.6), 687 (0.03), 605 (0.11), 579 (0.18), 551 (0.11), 417 (100), 375 3 (10), 286 (7), 271 (6), 147 (22), 133 (6), 131 (6), 105 (4), 69 (15).

Ferruginyl succinate (**17**): Colorless crystals; m. p. 85 – 87 °C; $[\alpha]_D^{20}$: + 59.1 (*c* 0.04, $CHCl_3$); IR (film): ν_{max} = 2966, 2848, 1752, 1133, 754 cm^{-1}; HR-MS (EI): *m/z* = 654.46440 (calcd. for $C_{44}H_{62}O_4$: 654.46484); EI-MS: *m/z* (rel. int. %) = 654 (2.7), 570 (1), 444 (4.3), 429 (1.2), 417 (0.4), 387 (0.3), 369 (18), 286 (100), 271 (45), 201 (20), 189 (21), 187 (13), 175 (21), 159 (49), 149 (13), 147 (16), 83 (14), 69 (39), 55 (25).

Supporting information

^{1}H-NMR data and ^{13}C-NMR data of compounds **2 – 17** are available as Supporting Information.

Results and Discussion

Ferruginol is an aromatic diterpene containing an abietane moiety. It was first obtained from *Podocarpus ferruginea* (Podocarpaceae) and proved to be a powerful gastroprotective agent [16]. In a previous report, a dose-response study of ferruginol showed that, at 25 mg/kg, the gastroprotective effect of the diterpene was similar to that of lansoprazole at 20 mg/kg in HCl/EtOH-induced gastric lesions in mice. Therefore, we chose the dose of 20 mg/kg to compare the effect of ferruginol with the new semisynthetic derivatives.

Starting from ferruginol (**1**), 16 derivatives (**2 – 17**) were obtained by semisynthesis. The ^{1}H- and ^{13}C-NMR spectroscopic data of these compounds are presented in **Tables 1S – 5S** (Supporting Information). All compounds prepared in this work present spectroscopic data in agreement with the proposed structures. Compounds **2 – 17** were not found in the literature and are described for the first time. The structures are presented in **Fig. 1**.

An investigation on the structure-gastroprotective effect of the diterpene dehydroabietic acid (DHA) and its semisynthetic derivatives was undertaken by Wada et al. [22]. In this study, 70 DHA derivatives at the position 12 and/or 18 were prepared and tested for a possible gastroprotective effect using the antisecretory and antipepsin assays in rats. Recently, Sepúlveda et al. [23] reported the gastroprotective effect of 19 DHA derivatives, assessed in the HCl/EtOH-induced gastric lesions model in mice at a single oral dose of 100 mg/kg. Several compounds presented a strong gastroprotective effect and some structure-activity trends were observed.

The derivatives **2 – 8**, with C_2 to C_{18} alkyl chains, included saturated and unsaturated fatty acids and two halogenated C_2 esters. In the C_2 esters with a halogen atom (compounds **2** and **3**), the chlorine derivative **2** reduced the lesion index by 51 % while the iodine derivative **3** was inactive. In the aliphatic esters **4 – 8**, the isobutyrate (**4**) was devoid of activity and the gastroprotective effect increased with the ester chain length as can be observed by comparing the activity of the laurate (**5**) with those of the palmitate (**6**) and oleate (**7**) derivatives. Regarding the fatty acid esters **5 – 8**, including both saturated C-12 and C-16 compounds (**5** and **6**) as well as unsaturated C-18 : 1 and C-18 : 2 products (**7** and **8**), the gastroprotective effects of the palmitate and oleate derivatives were similar to that of ferruginol but were decreased in the C-18 : 2 derivative **8**.

When comparing the effect of the newly prepared aliphatic esters with those described by Areche et al. [17], the isobutyrate ($C_{4:}4$) and the propionate (C_3) derivative tested by them were inactive but they observed that the presence of a chlorine atom in the C_3 ester chain or a double bond (propenoate) led to active derivatives. For C_2 and C_3 esters, we also observed that the presence of a chlorine atom is relevant for the gastroprotective effect. For fatty acid esters, our results showed that the effect appears to increase with longer side chains but unsaturation decreases the activity.

Considering the aromatic derivatives **9 – 12**, the best effect was elicited by the 3,5-dinitrobenzoate (**11**) and compound **12**, which showed a gastroprotective effects similar to that of ferruginol. The dinitro derivative **11** proved to be more active than the mononitro compounds and the *m*-substituted product was much more active than the *p*-substituted aromatic ester [17]. The salicylate **9** presented a similar gastroprotective effect as that of the benzoate described by Areche et al. [17], with lower activity for the phenylacetate **10**. The heterocyclic derivative **13** presented a significant gastroprotective effect, higher than that of ferruginol, being worthy of additional dose-response studies to find the corresponding EC_{50}. In the indoleacetate **14** and indolebutyrate **15** derivatives, differing in the chain length, the gastroprotective effects were similar, reducing lesions by around 50 %. A similar observation was made for the diesters **16** and **17**. Both compounds differ in the nature of the acid linkers (phthalic and succinic acid), suggesting that the gastroprotective effect does not depend on the identity of the selected diacids. The activity of compounds **1 – 17** in the gastric lesion model in mice is summarized in **Table 1**.

Regarding cytotoxicity, esterification with chloroacetic and iodoacetic acids (**2** and **3**) led to products with higher or similar cytotoxicity than the starting compound. The 2-chloroacetate derivative was more cytotoxic than ferruginol, presenting no selectivity towards both AGS cells and fibroblasts. Esterification of

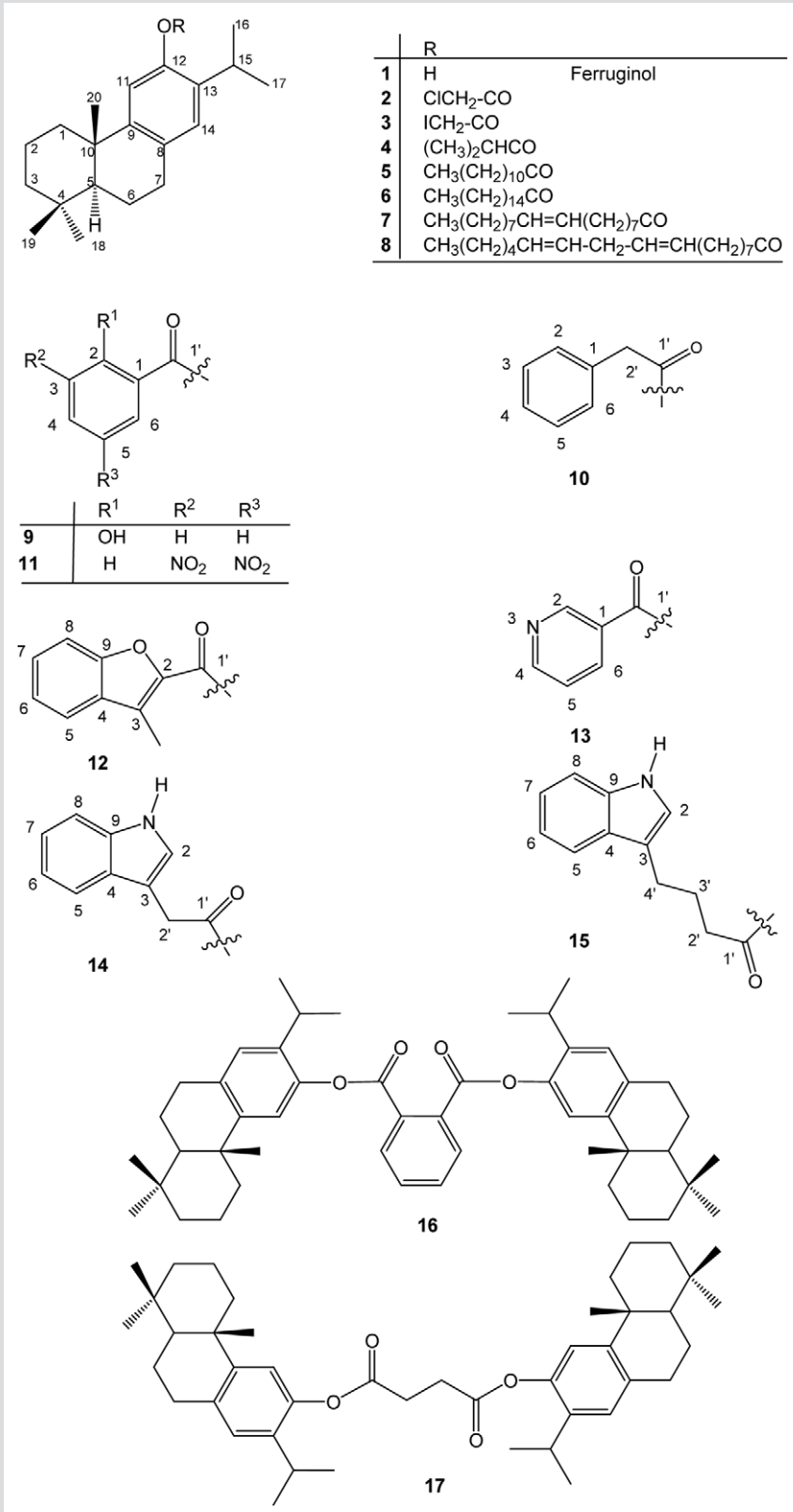

Fig. 1 Structures of ferruginol **1** and ferruginol derivatives **2 – 17.**

ferruginol with carboxylic acids (**5 – 8**) afforded less toxic derivatives with almost no effect on fibroblasts (IC_{50} >1000 μM). The isobutyrate, however, was highly selective with an IC_{50} >1000 μM and 60 μM towards fibroblasts and AGS cells, respectively. Among the aromatic derivatives **9 – 11**, the 3,5-dinitrobenzoate (**11**) presented remarkable selectivity with an IC_{50} of 29 μM against AGS cells, compared to >1000 μM for fibroblasts. The heterocycles **13 – 15** proved to be less cytotoxic on fibroblasts but had a strong and selective effect on AGS cells, particularly compounds **13** and **14** with IC_{50} values of 22 and 12 μM, respectively. Comparing the cytotoxicity on AGS cells of compounds **14** and **15**, differing only in one methylene (CH_2) be-

Table 1 Gastroprotective effect of ferruginol (**1**) and the semisynthetic derivatives **2 – 17** at the single oral dose of 20 mg/kg, and cytotoxicity (IC_{50}, μM) towards MRC-5 fibroblasts, AGS and Hep G2 cells

Compound	Lesion Index (mm)	% Lesion inhibition	Cytotoxicity IC_{50} (μM)		
			MRC-5	AGS	Hep G2
1	15.5 ± 7.7**	61	23.0	27.0	68.5
2	19.4 ± 6.9**	51	14.2	7.3	> 1000
3	30.4 ± 5.7	24	44.9	23.8	> 1000
4	30.7 ± 7.2	23	> 1000	59.7	39.2
5	23.3 ± 6.2*	42	> 1000	> 1000	> 1000
6	18.9 ± 5.1**	53	> 1000	567	519
7	18.3 ± 5.3**	54	> 1000	838	> 1000
8	23.8 ± 5.5*	40	> 1000	554	> 1000
9	24.7 ± 5.7*	38	> 1000	903	> 1000
10	27.8 ± 6.4*	31	> 1000	710	> 1000
11	13.4 ± 5.2**	66	> 1000	28.8	22.1
12	16.6 ± 5.5 **	58	> 1000	695	> 1000
13	11.7 ± 4.5**	71	> 1000	22.4	161
14	18.1 ± 5.1**	55	> 1000	11.5	315
15	20.1 ± 6.2**	50	> 1000	465	> 1000
16	20.5 ± 4.8**	49	> 1000	774	> 1000
17	21.3 ± 5.9*	47	773	455	637
Vinblastine			78	122	254
Lansoprazole	9.2 ± 4.8**	77	306	162	221
Control	39.9 ± 6.5				

* $P < 0.05$; ** $P < 0.01$; n = 7 – 8; mean ± S.D.

tween the aromatic ring and the carbonyl group, a higher selectivity can be seen for compound **14** (12 μM) than for compound **15** (465 μM). The diesters **16** and **17** were less toxic than ferruginol towards both fibroblasts and AGS cells, however, they were lacking selectivity.

Hep G2 cells can biotransform the compounds added to culture media because they express cytochrome P450, which is able to metabolize xenobiotics. For compounds **2**, **3**, **7 – 10**, **12 – 16**, lower cytotoxicity was found towards hepatocytes than towards AGS cells suggesting that the derivatives are being metabolized in the liver cells. The cytotoxicity of compounds **1 – 17** towards AGS cells, fibroblasts and hepatoma cells is summarized in ◉ **Table 1**.

When comparing the gastroprotective effect and cytotoxicity data, the most promising compounds are the derivatives **11**, **13** and **14**. The three compounds have a gastroprotective effect at least comparable to that of ferruginol, but with higher selectivities against the tumoral AGS cells with an IC_{50} of 12 μM for the indoleacetate **14**, followed by the nicotinate **13** (IC_{50}: 22 μM) and the 3,5-dinitrobenzoate **11** (IC_{50}: 29 μM). The IC_{50} values of these compounds on fibroblasts were >1000 μM. The three compounds bear an N atom either as an amino (indoleacetate and nicotinate) or nitro (3,5-dinitrobenzoate) function. The isobutyrate **4**, lacking a gastroprotective effect at 20 mg/kg is of interest because of its selective cytotoxicity against AGS cells (IC_{50}: 60 μM). Several derivatives showed low cytotoxicity values and good gastroprotective activity, suggesting that the two effects are not related. For aliphatic ester derivatives of ferruginol, the gastroprotective effect seems to be related to the side chain length. Better activity was found in the C_{16} and C_{18} derivatives but tended to decrease with increasing aliphatic chain unsaturation. For short-chain esters, the gastroprotective effect can be observed when the chain contains a chlorine atom. For aromatic esters, the presence of dinitro groups or a nitrogen atom in the aromatic ring enhances the gastroprotective activity. Additional studies including computational design should be undertaken to prepare more active derivatives and disclose the structure-relationship trends in this group of compounds.

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