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Detection of *CYP2C9*3* alleles by target-assembled tandem oligonucleotide systems based on exciplexes

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Cytochrome P450 (CYP) enzymes are important in the oxidative metabolism of both endogenous substrates and xenobiotics in the human liver. Genetic polymorphisms of P450 enzymes result in distinct sub-populations that differ in their ability to perform particular drug biotransformation reactions. The human CYP2C subfamily consists of four gene products: CYP2C8, CYP2C9, CYP2C18, and CYP2C19. CYP2C9 is the major CYP2C gene product that catalyses the metabolism of important drugs such as phenytoin, S-warfarin, tolbutamide, losartan and diclofenac in the human liver (Romkes et al 1991). At least 20 CYP2C9 Single Nucleotide Polymorphisms (SNP) alleles have been identified to date (http://www.imm.ki.se/CYPalleles). For example, CYP2C9*3 (1075 A⇒C) causes a change in the protein sequence from Ile to Leu at position 359, and is associated with decreased enzyme activity. In turn this results in increased sensitivity to drugs such as S-warfarin. Here we report the use of an exciplex-based split-probe system for detection of the wild type (WT) and *3 mutant alleles of human cytochrome P450 2C9. The detection system is split at a molecular level into signal-silent components (i.e. 2 sequence-specific oligonucleotide probes). On hybridization to their correct complementary target sequence, these components are assembled into a specific 3-dimensional structure, and can give an exciplex, which is detected by fluorescence spectroscopy. 8–mer or 12-mer synthetic oligonucleotides, labelled with pyrenyl or naphthyl exciplex partners at their 5′- or 3′- termini, respectively, were evaluated. Target DNA used in this study was in the form of short synthetic oligonucleotides, PCR products (150 bases) or cloned plasmid DNA (~3 kb). DNA was amplified and cloned from a plasmid template p17a2C9 which contains the human CYP2C9 wild type gene (courtesy of Dr J. Andrews, University of Manchester). The *3 mutant allele was constructed by PCR based mutagenesis of the wild type sequence. Using 8-mer or 12-mer split probe systems, annealed to a complementary WT oligonucleotide target (22- or 24-mer), led to characteristic exciplex fluorescence at 480 nm after excitation at 350 nm (Bichenkova et al 2005). This was accompanied by a quenching of and slight red shift in the pyrene signal. With *3 SNP target oligonucleotide, exciplex signals of lower fluorescence intensity were detected with both sets of probes, and there was a decrease in the T_m of the hybrid duplex from 20°C (WT) to 18.5°C (*3) with 8-mer probes and 37.6°C (WT) to 35.6°C (*3) with 12-mer probes. Using more complex DNA targets (PCR product and plasmid DNA) exciplex fluorescence was detected under certain circumstances on hybridisation with the probes, but not under other conditions. However, there was always a characteristic quench-

ing and slight red shift in the pyrene signal, indicating that the exciplex had formed, and melting curves obtained at the exciplex fluorescence wavelength were sigmoidal in shape, indicative of duplex formation. Detection of SNP mutations using this split-probe system gives a highly specific, simple, and accessible method to meet the rigorous requirements of pharmacogenomic studies. These results show that it is possible for the exciplex probe system to act as detectors for cytochrome P450 SNPs, with promise for future applications in genetic testing and molecular diagnostics.

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Evaluation of the antiplasmodial effect of retinol on *Plasmodium berghei berghei* infection in mice

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Malaria has been identified as a major threat to global health in the coming decades. Vitamin A is often deficient in individuals living in malaria endemic areas and several studies reveal an association between vitamin A and malaria and that it could play a part in potentiating resistance to malaria. A previous study found retinol to have in vitro activity against plasmodium falciparum at concentrations close to those in normal human serum Hamzah et al (2003). This study aims to evaluate the antiplasmodial activity of retinol on chloroquine-sensitive *Plasmodium berghei berghei* infection in mice. Male albino mice, 18–25 gm, were divided into three groups (A–C). The first two groups (A and B) were each divided into 5 subgroups of 10 mice per subgroup with one subgroup receiving the standard drug and another serving as control. The third group (C) consisted of just one subgroup of 10 mice. For Group A, the method described by Knight & Peters (1980) was used to determine the chemosuppressive effect of retinol on early infection. For Group B, the repository activity of retinol was evaluated using the method described by Peters (1965). Groups A and B received acute doses of retinol (50, 100 and 200 mg/kg) while Group C recieved chronic retinol doses (100 mg/kg daily, throughout the study period). The average percentage suppression of parasitaemia was calculated in comparison with control as shown below:

Retinol produced a mild, dose dependent schizontocidal effect on early and established *P. berghei* infection compared with the standard drug (Table 1). Chronic administration of retinol proved to be quite toxic to the parasites leading to their eventual clearance. However toxic manifestations were also observed in the animals leading to their death a few days after parasite clearance. Prophylactic administration of retinol caused a significant delay in the onset of infection compared with the control. It also caused a dose dependent repository activity at the various doses employed (Table 2). However the standard agent (pyrimethamine) produced a higher chemosuppression compared to the retinol treated groups. Retinol possesses antiplasmodial activity especially during chronic administration thus suggesting that it might have a role in malaria control. Further studies in the area of its structural activity relationships and an asessment of more potent synthetic retinoids are needed to justify this assertion.

Table 1 Blood schizonticoidal activity of different doses of retinol during early infection (4-day test)

Drug	Dose mg/kg/day	Average parasitaemia	Average % suppression
Retinol	200	5.43 ± 0.25*	52.78
	100	5.63 ± 0.15*	51.04
	50	6.77 ± 0.31*	41.13
Retinol vehicle (control)		11.50 ± 0.84	—
CQ (Std)	5	3.40 ± 0.23	70.43
One-way	ANOVA	F	2.74

Data expressed as mean ± s.e.m. for 10 mice per group df 4,45 $*P < 0.05$ when compared with control.
Early infection – determined 4 days post inoculation.
Equal volumes (0.1 ml) of drug and control vehicle were administered.

Table 2 Prophylactic effect of retinol against *P.berghei* infection (Repository test)

Drug	Dose mg/kg/day	Average parasitaemia (%)	Average suppression (%)
Retinol	200	3.45 ± 0.35*	46.9
	100	3.84 ± 0.35*	40.9
	50	4.86 ± 0.19*	25.2
Pyrimethamine	1.2	1.40 ± 0.21	78.5
Retinol vehicle (control)		6.50 ± 0.24	—
One-way	ANOVA	F	5.07

Prophylactic effect – determined 3 days post inoculation.
Equal volumes (0.1 ml) of drug and control vehicle were administered.

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Synthesis and ACE-inhibitory activity of a captopril nitroxoxyethylamide a novel hybrid prodrug of captopril and itramine

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The concept of coadministration of nitrates with other vasodilators such as captopril is well established in the literature (Fung et al 1993). The addition of a nitric oxide-releasing functionality to captopril in the form of a prodrug has been proposed previously (Ingram et al 2005). However, despite showing favourable in vitro activity it proved unsuitable in formulation studies. Therefore, we have developed a putative nitric oxide amide derivative that will be more resistant to degradation. Itramine (aminoethylnitrate) was used as a suitable nitrovasodilator due to its established pharmacological profile. The putative derivative captopril nitroxoxyethylamide (CNEA) was synthesised via the direct reaction of captopril and ethanolamine in the presence of N, N'-dicyclohexycarbodiimide to form captopril ethanolamide (CEA), this was then subjected to halogenation using thinoyl chloride and final nitroxylation using silver(I) nitrate to form the putative CNEA. The metabolic fate of CNEA in either in vivo or in vitro is unknown and is under investigation by this group. It is proposed that CEA is a likely metabolite. Therefore, we have examined the ACE-inhibitory effect of CNEA, CEA and compared with the parent drug, captopril. Captopril, CEA and CNEA were examined quantitatively for their ability to inhibit angiotensin I-induced contractions of rat aortae in-vitro. Thoracic aortae were obtained from male wistar rats, 170–230 g, and were cut into helical spirals 50–70 mm in length. Each spiral was mounted under 500 mg passive tension in Krebs ringer solution maintained at 37°C and gassed with 95% O_2 5% CO_2. Contractile responses to angiotensin I and II were recorded isometerically. Angiotensin II produced dose-related contraction which were unaffected by prior administration of captopril. Angiotensin-I induced contractions are, however, diminished in the presence of captopril, indicating the necessity of the conversion of angiotensin I to angiotensin II by endothelial ACE, and totally inhibited at 10^{-8} M captopril. CEA and CNEA were unable to achieve total inhibition at 10^{-8} M, both achieved 40% of the inhibition associated with captopril. This suggests that the CEA and CNEA retain some of the ACE-inhibitory properties of captopril despite the masking of the carbonyl functionality, while in vivo metabolism to captopril will result in full ACE inhibitory activity of the parent molecule.

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Effect of cytokine fragments and monocyte chemoattractants on the light-scattering properties of U937 cells

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The ability of leukocytes to change shape when exposed to chemoattractants and the correlation of cell shape change with changes in light-scatter of cells (Cole et al 1995) was used as the basis to evaluate two cytokine fragments, Platelet Factor 4 [47–70] (P) and Interleukin 1-beta [163–171] (β) together with three other known attractors of monocytes — formylmethionyl-leucyl-phenylalanine (fMLF), Lipopolysaccharide (LPS) and Casein. Cells from a human histiocytic lymphoma cell line, U937, were differentiated into granulocytes by treatment with dibutyryl cAMP for 48 hours before resuspension in a buffer containing Hanks Balanced Salt Solution, 1% Bovine Serum Albumin and 1mM HEPES (~10^5 cells/ml). The cells were then incubated with (1 ml) peptides (2×10^{-9} M to 2×10^{-5} M) or chemoattractants (LPS, 1 μg/ml and 20 ng/ml; fMLF, 2×10^{-8} M; Casein, 5 mg/ml) for 20 min (37°C, 5% CO_2). At the end of the incubation period the cells were fixed with glutaraldehyde and washed with phosphate buffered saline. This procedure was done in triplicate. After resuspension in BD FACSFlow the cells were analysed using a flow cytometer (BD FACSCanto). The side scatter (SSC) and forward scatter (FSC) of the 10 000 cells analysed for each treatment were displayed on a dot plot and the cells partitioned using polygon gates separating cells that have low FSC and SSC (G1, dead cells), intermediate FSC and SSC (G2, transformed cells), or high SSC (G3, large, transformed cells). The cell populations, median FSC and SSC values were then recorded and analysed. Increases in FSC, correlated with cell shape changes, were previously observed in neutrophils exposed to chemoattractants (Cole et al 1995). FSC changes of differentiated U937 cells (dU937), however, were variable even amongst standard chemoattractants (LPS 2.16% increase in G3 and 5.56% increase in G2, Casein 1.33% decrease in G3 and 5.26% decrease in G2). SSC changes, however, were more prominent and consistent with the highest change obtained for G3 cells treated with P at 2×10^{-7} M (260727 units vs. 243574 units, buffer treated only control). As the SSC for these cells treated with 1 μg/ml of LPS was similar (256257 units), this suggests that the level of cell activation with P at 2×10^{-7} M was on par with that of LPS. A better resolution of changes obtained with SSC compared to FSC for dU937 cells suggests that morphological changes and hence the ability to induce chemotaxis may be better tracked by observing for changes in the two light-scattering parameters rather than FSC alone. The ability to provide better morphological resolution using both FSC and SSC also been noted previously (Watson 1991). Another result which was unusual was the finding that in dU937 treated with 2×10^{-7} M of β, the G1 cell population number decreased when the overall trend for that population across all other treatments was an increase. This suggests that at that concentration, β may influence dU937 activation or apoptosis.

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In vitro adsorption of a range of drugs on two activated charcoal formulations

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Activated charcoal, genarally administered by mouth as a slurry in water, is capable of adsorbing a wide range of drugs and poisons by reducing the absorption of these toxic substances from the gastrointestinal tract. It therefore occupies a significant place in the treatment of acute oral poisoning (Martindale 2002). The adsorption process is influenced by numerous factors, including characterisics of the adsorbent (charcoal) (e.g. its physicochemical properties), namely particle size, the type of preparation (i.e. whether presented as a powder or slurry); by the chemical nature of the adsorbate(drug/poison) or by environmental conditions such as pH and temperature during the adsorption process (Terzyk et al 2003). For maximum efficacy, activated charcoal is administered within 1 h of ingestion of the toxic substance but can still be effective several hours after poisoning or in repeated dosing with drugs that either exhibit slow gastric emptying or those that are enterohepatically recycled. The aim and objectives of this study were to compare the response of two different activated charcoal preparations, Carbomix and Charcodote, to a range of commonly used drugs, to evaluate the rate and extent of adsorption and to examine the influence of pH on the adsorption process for a range of compounds. The drugs investigated were propanolol hydrochloride, theophylline, paracetamol, diclofenac sodium and ibuprofen. In 200 ml conical flasks, various concentrations of drug solutions were prepared each to a volume of 100 ml in water, 100 ml in simulated gastric fluid, GF (0.1 M HCl) or 100 ml in simulated intestinal fluid, IF(buffer, pH7.2) to which 50 mg of Carbomix powder or 4 ml slurry containing 50 mg activated charcoal, Charcodote, were added. Suspensions were agitated in a water bath at 37°C for 60 min after which samples were filtered, analyzed by UV, and equilibrium concentrations determined by a depletion method by comparison with calibration data for each drug. Adsorption isotherms (not shown) were constructed for each charcoal preparation with each drug in the various media and compared with the Langmuir and Freundlich models, equilibrium constants will be presented. A summary of the results in IF for Carbomix indicate that paracetamol, theophylline and propranolol hydrochloride exhibit Langmuir adsorption with rapid uptake at low equilibrium concentrations and reaching a plateau in adsorption for the three drugs between 8 and 20 ug/ml equilibrium concentrations. The rate of adsorption of diclofenac sodium and ibuprofen is rapid throughout the study and equilbrium is not reached for either drug in this medium with Carbomix. In contrast, it is the profiles of theophylline, paracetamol and ibuprofen which best fit the Langmuir model with monolayer saturation occuring at 10 ug/ml for all three drugs in IF with Charcodote; and there is sharp and rapid adsorption for propranolol hydrochloride and diclofenanc sodium in this medium without attainment of equlibrium. The results indicate that there are significant differences in the adsorption rate and extent depending on the media, charcoal formulation used, and nature of the drug compound and it may be concluded that the judicial choice of charcoal preparation administered in oral poisoning is dependent on the nature of the adsorbate.

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Effects of the phytoalexin, resveratrol, on the smooth muscle relaxant actions of tamoxifen

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Tamoxifen, a selective oestrogen receptor modulator (SERM), has been shown both to reduce the risk of breast cancer recurrence and, during the 5 years of recommended treatment, to protect women against acute myocardial infarction and angina (Bradbury et al 2005). A component of this cardiovascular protection is thought to be vasodilation. The natural product, resveratrol, which is found in red wine and grapes, possesses oestrogenic activity and also acts as a SERM. Unlike tamoxifen, which functions therapeutically as an antagonist, resveratrol is a weak agonist at oestrogen receptors (Harris et al 2005). Epidemiological studies have shown beneficial effects of red wine consumption on coronary disease, which is sometimes called the 'French Paradox'. The reduction in coronary disease is attributed to the resveratrol content of red wine. Since it is an oestrogen agonist, it is possible that women's consumption of resveratrol in wine could attenuate the beneficial dilator actions of tamoxifen. We previously showed that oestrogens elicit similar relaxation in both vascular and intestinal muscle (McCurrie et al 2004); in these experiments we investigated the effects of resveratrol on smooth muscle and its possible effect on the relaxant action of tamoxifen, using intestinal smooth muscle as a simple model. Segments of terminal ileum from Hooded-Lister rats (250–370 g) were set up in Krebs' solution (37°C, 95%O_2, 5% CO_2) containing 10 μM indomethacin under 1 g tension. Control concentration–response curves were constructed to carbachol (10 nM–30 μM) and repeated in the presence of one of the following: resveratrol (20–40 μM), tamoxifen (20–40 μM) or a combination of resveratrol (20 μM) plus tamoxifen (20 μM), N = 6. Resveratrol (20 μM) was weakly relaxant and shifted carbachol concentration- response curves rightwards, with little effect on Emax. Resveratrol (40 μM) caused further rightward shifts in the curve and a small reduction in Emax (10.9 ± 5.1%, NS). Tamoxifen (20 μM and 40 μM) was apparently a more potent relaxant agent, reducing Emax by 27.1 ± 6.2 and 66.2 ± 4.9%, respectively ($P < 0.05$). In combination, resveratrol (20 μM) plus tamoxifen (20 μM) shifted the carbachol concentration–response curves rightwards and reduced Emax by 33.1 ± 4.3%. No vehicle effects were observed (tamoxifen:80% ethyl alcohol/ 20% water, resveratrol:100% ethyl alcohol). These results demonstrate that the relaxant effects of tamoxifen in vitro are not attenuated by the presence of resveratrol; addition of resveratrol slightly increased the extent of relaxation observed. Our previous work has shown that rodent vascular and intestinal smooth muscle respond similarly to SERMS (McCurrie et al 2004). If human blood vessels respond in the same manner it is unlikely that consumption of red wine by women during treatment with tamoxifen would attenuate the beneficial vasodilator and cardioprotective effects of tamoxifen. However, there are many species differences in response between the various types of smooth muscle and further work will be necessary to confirm this outcome in the human subject.

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Analgesic, anti-inflammatory and antidiabetic effects of *Securidaca longepedunculata* (Fresen.) [Polygalaceae] root-bark aqueous extract

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Securidaca longepedunculata (Fresen.) [family: Polygalaceae] is widely distributed in tropical and subtropical parts of Africa, and occurs naturally in the North-West and Limpopo Provinces of South Africa. *S. longepedunculata* is usually a small, erect, perennial woody tree of up to six metres in height, with a pale-grey, smooth stem-bark (Watt & Breyer-Brandwijk 1962; Van Wyk et al 2002). The plant produces clusters of attractive pink or violet-purple flowers in early South African summer. The roots, but sometimes also the leaves and stem-bark of *Securidaca longepedunculata*, are commonly used traditionally in South African folk medicines for the treatment, management and/or control of an array of human ailments. This study was undertaken to investigate the analgesic, anti-inflammatory and antidiabetic properties of the plant's root-bark aqueous extract (SLE) in mice and rats. The experimental protocol and procedures used in this study were approved by the Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa. The analgesic effect of SLE (50–800 mg kg^{-1} i. p.) was examined in mice, using the 'hot-plate' and 'acetic acid' analgesic test models. Morphine (10 mg kg^{-1} i. p.) and diclofenac (100 mg kg^{-1} i. p.) were used as reference analgesic agents for comparison. The anti-inflammatory and antidiabetic effects of the plant's root-bark aqueous extract were investigated in rats. Fresh egg albumin-induced pedal oedema, and streptozotocin (STZ)-induced diabetes were used as experimental models of acute inflammation and diabetes mellitus to examine the anti-inflammatory and hypoglycaemic properties of SLE, respectively. Diclofenac (DIC, 100 mg kg^{-1} i. p.) and chlorpropamide (CPP, 250 mg kg^{-1} p. o.) were used respectively as reference anti-inflammatory and hypoglycaemic agents for comparison. *S. longepedunculata* root-bark aqueous extract (SLE, 50–800 mg kg^{-1} i. p.) produced dose-dependent, significant ($P < 0.05$–0.001) analgesic effects against thermally and chemically induced nociceptive pain in mice. *S. longepedunculata* root-bark aqueous extract (SLE, 50–800 mg kg^{-1} i. p.) also produced dose-related, significant reductions ($P < 0.05$–0.001) of the fresh egg albumin-induced acute inflammation of the rat hind paw oedema. Moreover, the plant's extract (SLE, 50–800 mg kg^{-1} p. o.) produced dose-dependent, significant reductions ($P < 0.05$–0.001) in the blood glucose concentrations of both fasted normal (normoglycaemic) and fasted STZ-treated, diabetic rats. The results of this experimental animal study indicate that *S. longepedunculata* root-bark aqueous extract possesses both central and peripheral analgesic effects, as well as anti-inflammatory and hypoglycaemic properties. These findings lend pharmacological credence to the suggested folkloric, ethnomedical uses of the plant's roots in the management, control and/or treatment of painful, arthritic and other inflammatory conditions, as well as for type 2, non-insulin-dependent diabetes mellitus (NIDDM) in some rural communities of South Africa.

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Actions of testosterone on rat portal vein

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Epidemiological evidence indicates that compared with age-matched males, premenopausal women have a reduced incidence of cardiovascular disease. It is likely that circulating oestrogen protects women's cardiovascular system, although testosterone could possibly act as a risk factor for cardiovascular disease. However, a number of investigations suggest that testosterone is a vasorelaxant agent: Rosano et al (1999) showed that in men with proven coronary artery disease, administration of testosterone improved tolerance to exercise-induced cardiac ischaemia, which could be attributed to a vasodilator action. The mechanism of testosterone-induced vasorelaxation remains controversial: involvement of vascular endothelium and nitric oxide (NO) production was suggested by Honda et al (1999), whereas Scragg et al (2004) concluded that testosterone acts via inhibition of L-type calcium channels in a manner similar to therapeutic dihydropyridines. In this work we investigated actions of testosterone on rat hepatic portal vein, in which the smooth muscle tested is not affected by vascular endothelium as it is separated from endothelium by a substantial circular muscle layer. Portal veins from male Hooded-Lister rats, 250–350 g, were placed under 0.5 g tension in Krebs' solution (37°C, 95%O_2, 5%CO_2). Concentration–response curves to KCl (10–100 mM) were constructed in the absence or presence of testosterone (TEST, 10–20 μM) or dexamethasone (DEX, 10 μM) (N = 6). To investigate possible involvement of NO, veins were equilibrated with either N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM), a nitric oxide synthase (NOS) inhibitor or the substrate for NO production, L-arginine (L-ARG, 100 μM). TEST(10, 20 μM) caused concentration-related reduction in responses to KCl, Emax being reduced by 34.4 ± 4.8% and 51.1 ± 8.4%, respectively. DEX was without effect on responses to KCl. Unexpectedly both L-NAME and L-ARG reduced relaxant effects of TEST (10 μM). Following incubation with L-NAME (100 μM), the reduction in KCl Emax induced by TEST decreased from 39.2 ± 5.2% to 27.3 ± 4.7%: in the presence of L-ARG(100 μM), a substrate for NOS enzymes that might be expected to enhance relaxation induced by TEST, Emax was reduced to 25.0 ± 4.1% (N = 4–6). To investigate possible calcium antagonism of TEST, veins were incubated in a calcium-free Krebs' solution. KCl (30 mM) was added to depolarise the tissue: after 5 min, concentration–response curves to calcium (10 μM to 20 mM) were constructed in the absence or presence of TEST (10–20 μM) or nifedipine (2–5nM), N = 4. TEST(10, 20 μM) reduced the calcium-induced responses, decreasing Emax by 26.5 ± 11.2% and 30.0 ± 7.3%, respectively. Whereas nifedipine (2, 5 nM) shifted the calcium concentration–response curves rightward causing a concentration-dependent reduction in Emax of 37.5 ± 7.2% and 70.7 ± 6.1%, respectively. These results show that relaxation is not a general property of steroids, since dexamethasone was without effect. The endothelium does not affect the longitudinal muscle of portal vein and although NOS inhibition reduced TEST-induced relaxation, this can be attributed to non-specific effects of L-NAME, since L-arginine, a NOS substrate, produced comparable effects. Similarly, although TEST reduced contractile responses to calcium, this was not dose-related and did not resemble the action of the dihydropyridine, nifedipine. We conclude that neither of the mechanisms proposed, endothelial or other tissue NO production or calcium antagonism, can account for the relaxant action of testosterone in rat portal vein.

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Colon specific mutual azo prodrug of 5-aminosalicylic acid: synthesis and pharmacological evaluation of its mitigating effect on TNBS induced experimental colitis

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Ulcerative colitis and Crohn's disease are categorised as inflammatory bowel disease (IBD). These are recurrent disorders chronically involving the mucosa and submucosa of the colon. 5-Aminosalicylic acid (5-ASA) is a widely prescribed drug for the long-term maintenance therapy of IBD. However, when 5-ASA is administered orally, a large amount of the drug is absorbed from the upper gastrointestinal tract (GIT) and causes systemic side effects. Therefore, it is preferable to deliver the drug specifically to colon. The prodrug approach is one of the significant strategies for drug targeting to colon. Sulfasalazine, olsalazine and balsalazide are prodrugs of 5-ASA, but none of them are free from adverse effects, which are due to the carriers used in their design. Therefore, the need for a safe prodrug of 5-ASA still remains. This work reports synthesis of a mutual azo conjugate of 5-ASA with an essential amino acid L-tryptophan with an objective of determining the efficacy of amino acid carrier system in delivering 5-ASA to colon, enhancing its mitigating effect on colonic inflammation while decreasing its ulcerogenic potential. An azo prodrug of 5-ASA with L-tryptophan (ST) was synthesized by coupling diazonium salt of amino acid methyl ester hydrochloride with salicylic acid (Furniss et al 1978). Its structure was characterized by elemental analysis and spectral data. The aqueous solubility and log P value of ST was found to be 0.28 g/ml and 0.26, respectively. The log P of ST is much lower than that of 5-ASA (0.64). In vitro release was evaluated in 0.05 M hydrochloric acid buffer (pH 1.2) and phosphate buffer (pH 7.4) over a time span of 4 h and 7 h, respectively at 37 ± 1°C. A negligible release was observed in both the buffers. In rat fecal matter, ST followed zero order kinetics with t of 143.6 min, K of $4.82 \times 10^{-3} \pm 0.0001$with 87.9% cumulated release of 5-ASA over a period of 7 h. Biological evaluation of the ST was carried out in Poona College of Pharmacy and its animal facility is approved by CPCSEA. The experimental protocols for the same have been approved by the Institutional Animal Ethical Committee. Ulcerogenic tendency was determined by Rainsford's cold stress method (Rainsford & Whitehouse 1975). Orally administered 5-ASA showed maximum ulcer index (59.6 ± 4.7). ST showed comparable lowering of ulcer index as that of sulfasalazine (9 ± 2). Therapeutic efficiency of the carrier system was studied in trinitrobenzene sulfonic acid (TNBS) induced colitis (Yamada et al 1992). All drug-receiving groups showed a decrease of inflammation severity after a lag time of 24–48 h. The decrease in clinical activity caused by ST was 1.06 ± 0.51 which is comparable with that caused by sulfasalazine (0.83 ± 0.42) in contrast to 2.09 ± 0.27 by 5-ASA. Tryptophan contributed positively towards the ameliorating effect of plain 5-ASA by lowering the clinical activity score rate to 1.83 ± 0.33. The histopathological features indicated that the morphological disturbances associated with TNBS administration were corrected by ST treatment and were comparable with sulfasalazine-treated group. The results demonstrate that this mutual prodrug has a remarkable ameliorating effect on the disruption of colonic architecture and suppresses the course of TNBS induced colitis effectively.

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Comparison of alternative methods for the assessment of gastric injury in rats treated acutely with non-steroidal anti-inflammatory drugs (NSAIDs) and their glyceryl esters

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Gastrotoxic effects of NSAIDs have driven the development of alternative formulations and chemical derivatives, including nitric oxide (NO) donors. Development of assays for the assessment of mortality and morbidity from NSAIDs has occurred alongside those for anti-inflammatory, anti-pyretic and analgesic efficacy (Khan 2004). Here we compared 4 alternative histological assays for quantification of stomach injury following acute oral treatment with drugs under appropriate Home Office licences. 49 male Wistar rats (7 replicates per treatment) were fasted for 16 h then treated by gavage with a single oral dose of one of the following alternatives: Parent NSAIDs, ibuprofen (0.52 mmol/kg) or indomethacin (0.08 mmol/kg), the respective nitric oxide glyceryl adducts or glyceryl esters, all at equimolar dose, or vehicle only (10% DMSO and 1% methylcellulose aq.). After 4 h animals were culled, stomachs everted, rinsed and injury scored by assay 1, a subjective macroscopic (SubMac) visual inspection of gut surfaces (Duffy et al 2001). Whole mount macro fields of view were digitally photographed, avoiding deliberate imaging of sites of injury, providing assay 2, an objective macroscopic (ObjMac) score of relative surface area of red pixels (values between 0–169 on a 0–255 scale) carried out using Image-Pro Plus (IPP). Tissues were fixed in 4% paraformaldehyde for 4 h, washed, cryoprotected in buffered 30% sucrose, stored at –20°C. Vibratome sections (50 microns) taken of identical sites were re-cryoprotected and stored frozen until defrosted in batches of complete treatment sets for reaction by diaphorase histochemistry, washed and mounted in glycerol under cover slides then digitally photographed under a ×10 objective. In assay 3, objective microscopic (ObjMic) measurements were made of mucosal thickness (micrometers) in cross section at unfolded portions of tissue using IPP software. In assay 4, subjective microscopic (SubMic) visual inspection was performed by a present/absent quantification of multiple signs including the presence of unstained patches of mucosal surface and abnormal mucosal edges not yet detached. Scores for each treatment are abstracted and expressed as mean ± s.e.m. for assay 1 only (Table 1). The null hypothesis was rejected by Minitab one-way analysis of variance ($P < 0.01$). Tukey's pair wise comparisons identified the following: Firstly, ibuprofen treatment caused a significantly greater level of injury compared with vehicle (*) NO-ibuprofen and glyceryl-ibuprofenate (#), indicating protection by ester and NO-adduct. NO-ibuprofen appeared to exert significant protection over glyceryl-ibuprofenate treatment. Secondly, indomethacin treatment also caused a significantly greater level of injury compared with vehicle (*) NO-indomethacin and glyceryl-indomethacinate (§), indicating protection by ester and NO-adduct. While assays 2 and 4 closely reflected this pattern, assay 3 of mucosal thickness was not able to resolve differences. We speculate that sampling to avoided mucosal folds overlooked major sites for injury or did not adequately discriminate sloughing.

Table 1 Mean gastric injury score by assay 1

Treatment	Visual score (Arb units)	ANOVA
Vehicle	1.0 ± 0.2	
Ibuprofen	16.2 ± 1.2	*
NO-Gly-Ibuprofen	2.8 ± 1.9	#
Glyceryl-Ibuprofenate	8.0 ± 0.4	*#
Indomethacin	14.5 ± 1.1	*
NO-Gly-Indomethacin	3.2 ± 1.2	§
Glyceryl-Indomethacinate	4.1 ± 1.6	§

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Nitrergic cells in the thymus of Dark Agouti rats treated chronically with antagonists of alpha-1- (urapidil) and beta- (propranolol) adrenoceptors

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The abundance of medullary thymic cells expressing inducible nitric oxide synthase, iNOS, termed nitrergic, is proposed to contribute to the negative selection of self-reactive thymocytes (Downing et al 1998) thereby limiting T-cell dependent autoimmune disorder. Deletion of self-reactive T-cells within thymus is incomplete; a less stringent tolerance mechanism would support a diverse T-cell repertoire that would benefit resistance to infection. However, some autoimmune prone animal strains express a relatively low nitrergic cell abundance of more than 2-fold compared to autoimmune resistant strains (Downing et al 1998; Azam et al submitted). Epigenetic, endocrine and neural controls of immune functions are of interest in the understanding of inflammatory disorder. Sympathetic nerves innervate primary lymphoid organs (thymus and bone marrow) entering the thymus parenchyma forming varicose plexuses in the subcapsular cortex and corticomedullary junction. Sparse sympathetic nerve fibers have also been found in the medulla. Although conventional synapses have not been observed there is ultrastructural evidence of sympathetic varicosities associated with both thymocytes and nonlymphoid thymic cells. Thymus contains predominantly beta-2-ARs but the regulated expression of alpha-1-ARs has been recognised (reviewed in Pleaš-Solarovi et al 2005). Chronic blockade of alpha-1-ARs by urapidil (Pleaš-Solarovi et al 2005) or β-ARs by propranolol (Leposavi et al 2006) affect thymic structure and thymocyte differentiation that we hypothesised could involve iNOS. Catecholamines (epinephrine and norepinephrine) are also known to promote the induction of iNOS synthesis and NO production by macrophages (Chi et al 2003). We therefore compared nitrergic cell abundance in 3-month-old male Dark Agouti rats treated by s.c. injection for 15 d with alpha-1-blocker (urapidil 0.02 mg/100 g/day), beta-blocker (propranolol 0.40 mg/100 g/day) or vehicle (an equivalent volume of saline). Paraformaldehyde-fixed thymi were stored frozen (–20°C) before parallel processing of batches containing all 3 groups for enzyme histochemistry in 100-micron sections. Abundance of medullary nitrergic cells stained positive by NADPH-diaphorase was used as a marker of inducible nitric oxide synthase (iNOS). Counts and section surface area measurements were made from glycerol-mounted sections, where tissue shrinkage is less than other studies using dehydration and xylene-based mountant. An observer blind to treatment performed the counts. Mean nitrergic cell abundance (count/mm^2) ± s.e.m. was 2.278 ± 0.334 (n = 8) for urapidil, 2.163 ± 0.230 for propranolol (n = 8) and 2.574 ± 0.378 for saline (n = 8). Although chronic adrenergic blockade appeared to decrease mean nitrergic cell abundance by 11.5% for urapidil and by 16% for propranolol, as might be expected if catecholamines promoted iNOS, one-way ANOVA with Tukey's follow up test failed to show significant difference with abundance in saline treated animals ($P = 0.650$). Counts by a second observer were concordant. We cannot rule out that small changes in the stringency of thymic selection (relative to those observed in autoimmune-prone strains) could be dependent upon autonomic mediation, but these might not be resolved by our small sample size. It is also known that glucocorticosteroids can decrease nitrergic cell abundance. If administration stress induced their release this may have occluded inhibitory effects hypothesised for adrenergic blockade.

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Relative abundance of nitrergic cells in the thymus of non-obese diabetic (NOD) mice compared with autoimmune resistant Balb C

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The non-obese diabetic (NOD) mouse has been used extensively as a preclinical tool in the development of new therapeutic strategies because of its similarities to type 1 diabetes in human (Anderson & Bluestone 2005). Various explanations exist for the underlying cause of susceptibility, including defects within thymus that might allow islet-reactive T cells to escape deletion and prevent the proper generation of regulatory T cells. The abundance of medullary thymic cells expressing inducible nitric oxide synthase, iNOS, termed nitrergic, is proposed as one mecha-

nism involved in the negative selection of self-reactive thymocytes (Downing et al 1998) thereby limiting T-cell dependent autoimmune disorder such as beta cell destruction. We therefore compared nitrergic cell abundance in prediabetic male and female mice from susceptible (NOD) and resistant (BalbC) mice. A total of 16 mice were used of the following ages (days): 4 male NOD (63 d); 4 female NOD (49 d); 4 male BalbC (49 d); 4 female BalbC (5 6 d). Beckman glucose Analyzer measurements of blood confirmed all animals as non-diabetic (glucose < 200 mg/dl). Paraformaldehyde-fixed thymi were stored frozen (−20°C) before parallel processing of batches containing all 4 groups for enzyme histochemistry in 100 micron sections. Abundance of medullary nitrergic cells stained positive by NADPH-diaphorase was used as a marker of inducible nitric oxide synthase (iNOS). Counts and section surface area measurements were made from glycerol-mounted sections, where tissue shrinkage is less than other studies using dehydration and xylene-based mountant. An observer blind to group performed the counts. Mean nitrergic cell abundance (count/mm^2) ± s.e.m. was 0.859 ± 0.245 for male NOD, 0.755 ± 0.165 for female NOD, 1.739 ± 0.252 for male BalbC and 1.954 ± 0.339 for female BalbC. Two-way ANOVA with Tukey's follow up test identified significant difference between strain ($P < 0.001$) but not gender. Counts by a second observer were concordant. Although the incidence of autoimmune diabetes in female NOD mice (60–80%) is reported to be greater than males (20–30%) (Anderson & Bluestone 2005) the susceptibility of gender was not associated with differences in medullary nitrergic cell abundance. However, the autoimmune-susceptibility of the NOD mice strain was found to be associated with a greater than 2-fold deficiency (measured by two independent observers) in medullary nitergic cell abundance compared with the resistrant Balb C strain. That neither NOD nor BalbC animals used were assessed yet to be diabetic suggests that the deficiency in nitrergic cell abundance could preceed autoimmune insulinitis. Tissues remain for analysis of pancratisis. Discerpancies in the age of animals used might have contributed in NOD to equalising nitrergic cell abunance in aged males compared to younger females, but this was not supported by age differences in BalbC and did not explain strain difference. Results are consistent with observed deficiency of medullary nitrergic cells in the autoimmune-susceptible Lewis rat compared with both Fischer and Sprague-Dawley, resistant strains (Downing et al 1998). We speculate that abnormality in nitric-oxide dependent mechanism(s) of central thymic tolerance and/or regulatory T cell production contribute to autoimmune susceptibility.

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Exploring antioxidant potential of triphala: a well known phytomedicine from Ayurveda

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Triphala' is one of the age old, most commonly used poly herbal preparation in Indian System of Medicine (ISM) particularly in Ayurveda. This well known phytomedicine is made in combination with *Terminalia chebula, Terminalia belerica* and *Emblica officinalis*, in equal proportions as reported in the Ayurvedic Formulary of India (AFI) (Anonymous 2002). This formulation is prescribed as laxative, detoxifying agent and rejuvenator in Ayurveda. Its anti-diabetic, antimutagenic, purgative, and radioprotective effect has been reported (Anonymous 2002). The individual herbs, used in the formulation are reported to have several other health benefits. Gallic acid (GA) is a common phytoconstituent present in all the three herbs used in the Triphala and is reported to possess hepatoprotective and antioxidant property, so the quantification of GA can be helpful in routine quality control of the Triphala and its different constituents. Triphala and its individual constituents were standardized with the High Performance Thin Layer Chromatographic technique using gallic acid (GA) marker compound (Mukherjee 2002). The GA content in Triphala with its individual constituents like *Emblica officinalis, Terminalia chebula* and *Terminalia belerica*, was found to be 28.14, 26.61, 27.22 and 27.18% w/w. Antioxidant activity of alcoholic extract Triphala (TAE) and its constituents was studied by in vitro and in vivo models. TAE and extracts of its constituents exhibited strong antioxidant activity as shown by the low IC_{50} values through in-vitro TBARS, hydroxyl radical inhibition assay, DPPH and nitric oxide methods. The values were found to be less to those of quercetin, the standard used. Administration of TAE at 100 and 200 mg kg^{-1} body weight given for five days before carbon tetrachloride (CCl_4) treatment caused a significant increase in the level of superoxide dismutase (SOD) and catalase and a significant decrease in the level of thiobarbituric acid reactive substances (TBARS), when compared with CCl_4 treated control in both liver and kidney (Rai et al 2006). These changes observed at 100 mg kg^{-1} body weight treatment were comparable with those observed for standard vitamin E at 50 mg kg^{-1} treatment. The results support significant antioxidant potential of TAE.

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