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STOMACH

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Background and aims: Nucleotides form the building blocks of DNA and are marketed as dietary supplements, alone or in combination with other ingredients, to promote general health. However, there has been only limited scientific study regarding the true biological activity of orally administered nucleotides. We therefore tested their efficacy in a variety of models of epithelial injury and repair.
Methods: Effects on proliferation ([^{3}H] thymidine incorporation) and restitution (cell migration of wounded monolayers) were analysed using HT29 and IEC6 cells. The ability of a nucleotide mixture to influence gastric injury when administered orally and subcutaneously was analysed using a rat indomethacin (20 mg/kg) restraint model.
Results: In both cell lines, cell migration was increased by approximately twofold when added at 1 mg/ml ($p<0.01$); synergistic responses were seen when a mixture of nucleotides was used. Cell proliferation was stimulated by adenosine monophosphate (AMP) in HT29, but not in IEC6, cells. Gastric injury was reduced by approximately 60% when gavaged at 4–16 mg/ml ($p<0.05$), concentrations similar to those likely to be found in consumers taking nucleotide supplements. Systemic administration of nucleotides was unhelpful.
Conclusions: Nucleotides possess biological activity when analysed in a variety of models of injury and repair and could provide a novel inexpensive approach for the prevention and treatment of the injurious effects of non steroidal anti-inflammatory drugs and other ulcerative conditions of the bowel. Further studies on their potential benefits (and risks) appear justified.

Natural medicinal products have been used for millennia for the treatment of multiple ailments. Although many have been superseded by conventional pharmaceutical approaches, there is currently a resurgence of interest in the use of natural bioactive products by the general public, with many healthy subjects and patients taking them for the prevention and treatment of multiple conditions, including gastrointestinal disorders and postoperative recovery.[1] Unfortunately, current evidence of the scientific validity of many of these traditional and commercial compounds is severely limited.

Nucleosides and nucleotides (NTs) serve as building blocks for RNA and DNA synthesis for cells. Cell turnover is very rapid in the gut and requires considerable amounts of NTs. The intestine can synthesise NTs from amino acids and other precursors but de novo NTs synthesis may be limited and the gut may also be dependent on exogenous supplies of NTs.[2] Therefore, a dietary source of NTs may optimise the function of rapidly dividing cells, such as those of the gastrointestinal tract, especially during periods of starvation or stress.

NTs are currently sold in pure individual form (particularly adenosine based), as NT mixtures, or as part of multiple ingredients in dietary supplements in health food shops. Multiple claims for these products have been made, including improving gastrointestinal and liver health, strengthening the immune system, promoting tissue renewal and wound healing, and benefiting athletic performance. The strength of the data supporting these claims are however limited.

We have therefore performed a series of studies to analyse the effects of NTs, given individually and in combination, with regard to mechanisms of gut integrity and repair using well validated in vitro and in vivo models.

MATERIALS AND METHODS

All chemicals were purchased from Sigma (Poole, Dorset) unless otherwise stated.

Ethics

All animal experiments were approved by the Imperial College School of Medicine Animals Ethics Committee and covered by the appropriate licences under the Home Office Animals Procedures Acts, 1986.

Study series A: effect of nucleotides on in vitro models of repair

Background to methods

One of the earliest biological repair responses following injury to tissue cells is migration of surviving cells over the denuded area caused by the injury to re-establish epithelial integrity. As it is extremely difficult to study this effect on organ tissue inside a human or animal, cell culture models are commonly used as surrogate markers of this promigratory response. Approximately 24 hours after the injury has occurred, there is also an increase in the rate of cell division in order to re-establish a normal mucosa. Cell culture models have traditionally also been used as surrogate markers for this proliferation response, and because thymidine is a natural constituent of DNA,[^{3}H]thymidine incorporation is commonly used as a marker of proliferation. Cells that are actively dividing will therefore increase their uptake of thymidine in the preparatory state of cell division.

Cell migration as a model of wound repair

Cell migration assays were performed using our previously published methods.[3] Briefly, human colonic carcinoma (HT29) and rat intestinal epithelial (IEC6) cells were grown to confluence in six well plates in medium consisting of

Abbreviations: AMP, adenosine monophosphate; CMP, cytidine monophosphate; GMP, guanosine monophosphate; IMP, inosine monophosphate; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; NSAID, non-steroidal anti-inflammatory drugs; NT, nucleotides

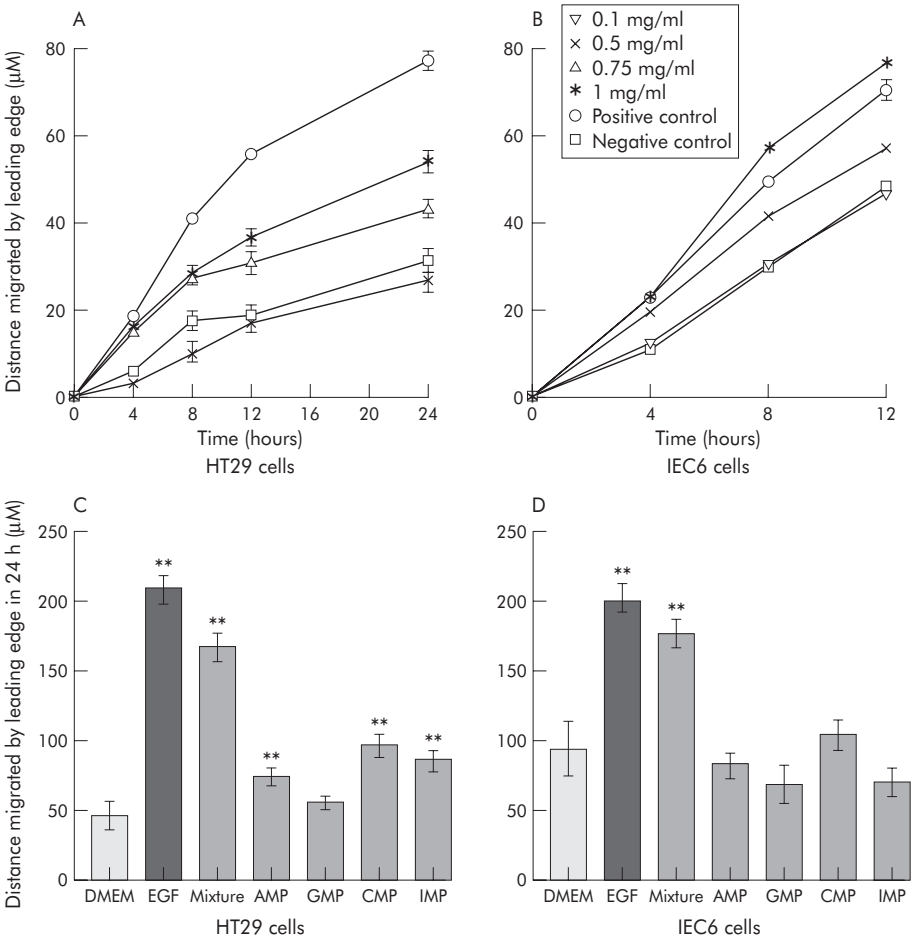

Figure 1 Effect of nucleotides (NTs) on wound healing, as assessed by cell migration. Standard wounds were inflicted on monolayers of (A) the human colonic cell line HT29 or (B) rat intestinal epithelial IEC6 cells. The degree of movement at various time points after wounding was then assessed in the presence or absence of various test factors. Addition of various concentrations of an NT mixture (0.1, 0.5, 0.75, and 1 mg/ml) stimulated a dose dependant increase in the rate of migration of the cells. Maximal stimulation was seen at 1 mg/ml for both cell lines. A negative control (Dulbecco's modified Eagle's medium (DMEM) alone) and a positive control (epidermal growth factor (EGF) 10 μg/ml) were also tested. $p<0.01$ versus control for all times after four hours for all doses of NTs in both cell lines. (C, D) Results of the distanced travelled at a single time point (24 hours) after injury. (C) Addition of the single NTs adenosine monophosphate (AMP), cytidine monophosphate (CMP), or inosine monophosphate (IMP) (1 mg/ml) stimulated increased movement in HT29 cells compared with the negative control (DMEM alone). Guanosine monophosphate (GMP) had no significant effect. The most pronounced promigratory effect was seen when an equal mixture of NTs was used at a total final total concentration of 1 mg/ml. EGF (10 μg/ml) was used as a positive control. (D) Addition of the single NTs AMP, GMP, CMP, and IMP(1mg/ml) had no significant effect on stimulated restitution in IEC6 cells compared with the negative control (DMEM alone). In contrast, addition of a mixture of NTs, also at a total final concentration of 1 mg/ml, markedly stimulated the rate of migration. EGF (10 μg/ml) was used as a positive control. **$p<0.01$ versus negative control.

Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C in 5% CO_2. The monolayers were then wounded by scraping a disposable pipette tip across the dishes, washed twice with fresh serum free medium, and cultured in serum free medium in the presence of individual NTs (adenosine monophosphate (AMP), cytidine monophosphate (CMP), guanosine monophosphate (GMP), inosine monophosphate (IMP)) alone or in combination. Additional monolayers containing a mixture of equal amounts of each NT, at a total final concentration of 1 mg/ml, also had the proliferation inhibitor, mitomycin C, added at 5 μg/ml in order to examine whether wound closure was dependant on cell proliferation. The rate of movement of the anterior edges of the wounded monolayers was then determined by taking serial photomicrographs at various times after wounding.[3] An inverted microscope (Nikon TS100) and a Nikon Coolpix 800 digital camera with 125-fold magnification were used to obtain photomicrographs. Identical regions were examined at each time point by pre-marking the base of the plates to facilitate alignment. Twenty measurements per field were performed by placing a transparent grid over the photograph and measuring the distance moved from the original wound line. All results are expressed as the mean (SEM) of three separate experiments.

Cell proliferation

Cell proliferation assays were performed using our previously published methods.[4] Briefly, IEC6 cells and HT29 cells were grown in DMEM containing glutamine and 10% fetal calf serum. Effects of NT and epidermal growth factor (EGF, positive control) were subsequently tested under serum starved conditions. To assess the rate of cells entering DNA synthesis,[^{3}H]thymidine (2 μCi/well) was included 24 hours after addition of the test factors and cells were left for a further 24 hours. For each condition, the stimulatory or inhibitory effect of the solutions was measured in quadruplet in six separate wells.

Study B: effect of the nucleotide mixture on an in vivo rat gastric damaging model

Background to method

Although cell culture studies provide valuable information regarding potential bioactivity, additional information may

also be gained by extending studies to the in vivo situation. The ability of NTs to prevent gastric damage in rats stressed by indomethacin and restraint was therefore also assessed using a well validated model.[3 5]

Method

Male Sprague Dawley rats (225–275 g; Harlan Olac Ltd, UK) were housed in standard cages (four animals per cage) and fed standard laboratory chow (Special Diet Services, Essex, UK) and tap water ad libitum.

Rats were randomised to receive, by gastric gavage, one of the following: 1 ml of saline alone or varying concentrations of the NT mixture (1–16 mg/ml) in saline. Additional animals received intragastric EGF at a concentration of 25 μg/ml (positive control). As in previous studies using this model, all gastric solutions also contained 2% hydroxypropylmethylcellulose to reduce the rate of gastric emptying.

Thirty minutes after gavage, all animals were placed in Bollman-type restraint cages and received indomethacin (20 mg/kg subcutaneously). Three hours later, animals were killed by stunning and cervical dislocation. The stomachs were then removed, the pH of the gastric contents measured, the stomachs inflated with 4 ml of 10% formalin, and stored overnight in 10% formalin. Stomachs were subsequently randomly coded and macroscopic and microscopic assessments of injury assessed in a blinded fashion. Macroscopic injury was assessed using a dissecting microscope (×10) with the aid of a reference square grid and reported as the total area of ulceration per stomach (mm^2/stomach). The stomachs were then embedded in wax and the depth of damage assessed microscopically as previously described.[5] Briefly, microscopic injury was graded with a score from 0 to 4 where 0 = no damage, 1 = one small erosion (less than 0.5 mm), 2 = two small or one large erosion (>0.5 mm),

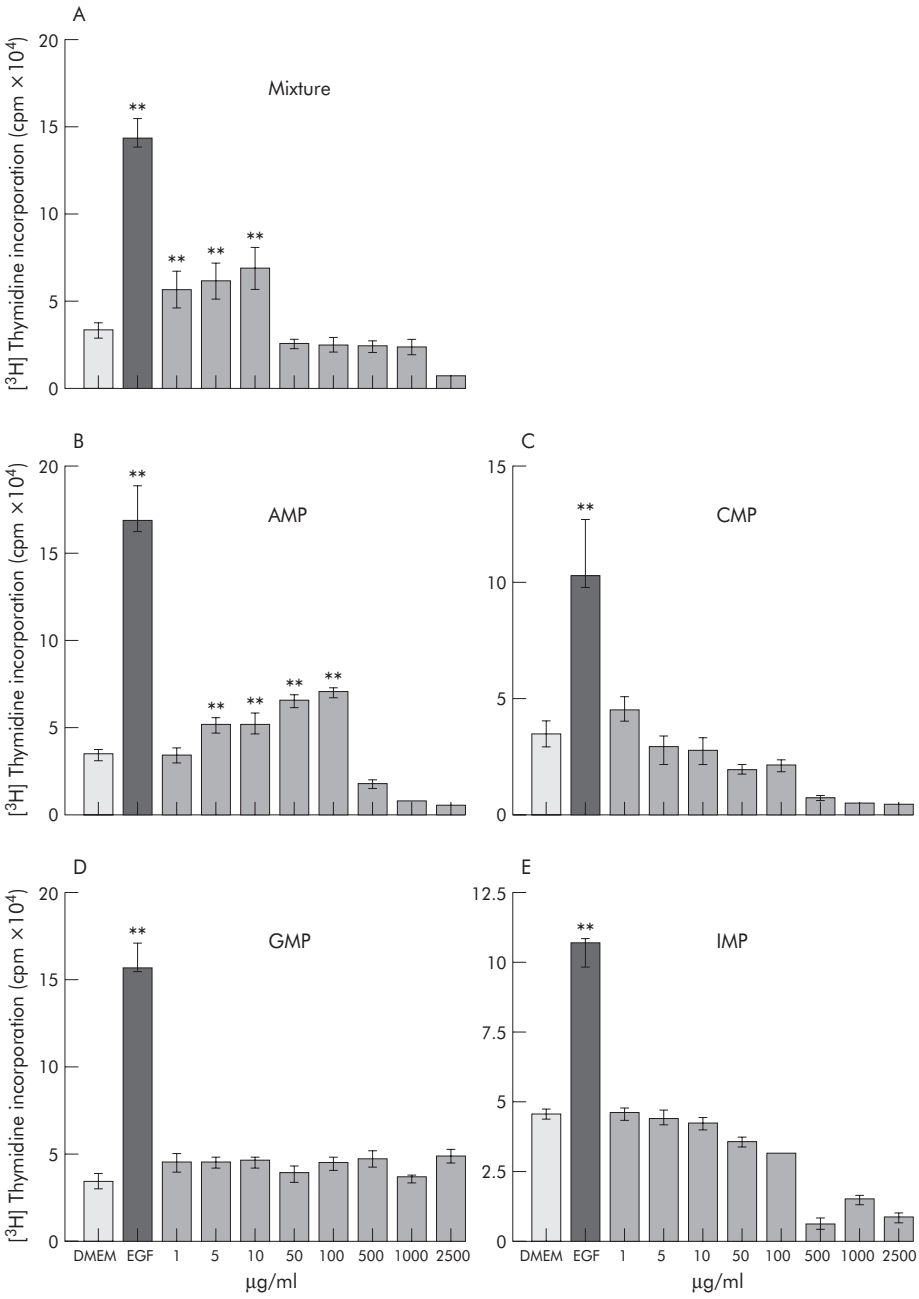

Figure 2 Effect of nucleotides (NTs) on proliferation of human colonic carcinoma HT-29 cells. (A) Addition of the mixture of NTs stimulated proliferation in a dose dependent manner (1–10 μg/ml) compared with the negative control (Dulbecco's modified Eagle's medium (DMEM) alone). Concentrations higher than10 μg/ml had no effect on, or reduced the amount of, proliferation. (B) Studies of individual NTs showed that adenosine monophosphate (AMP) stimulated proliferation in a dose dependent manner compared with the negative control (DMEM alone). Addition of cytidine monophosphate (CMP) (C), guanosine monophosphate (GMP) (D), and inosine monophosphate (IMP) (E) had no effect or reduced the level of thymidine uptake compared with the negative control. Epidermal growth factor (EGF 10 μg/ml) was used as a positive control. **$p<0.01$ versus negative control.

3 = two or more large erosions, and 4 = any area of ulceration extending to the muscularis mucosa.

In addition, in order to examine the importance of the route of administration on any effects seen, an additional series of rats were treated in a similar way but received test substances (saline or 2, 4, or 8 mg/kg/h of the NT mixture) via a continuous subcutaneous infusion rather than via gavage. Additional animals received subcutaneous infusions of EGF at a concentration of 5 μg/kg/h (positive control).

Statistics

All values are expressed as mean (SEM). One or two way ANOVA was used as appropriate. Where a significant effect was seen ($p<0.05$), individual comparisons were performed using *t* tests based on group means, residual, and degrees of freedom obtained from the ANOVA, a method equivalent to repeated measures analyses.

RESULTS

Cell migration as a model of wound repair

Addition of the NT mixture to either HT29 or IEC6 cells resulted in a significant ($p<0.05$) dose dependant increase in cell migration (fig 1A, B). Maximal stimulation was seen at 1 mg/ml; higher doses did not enhance migration further (data not shown).

Cells which had been incubated with the NT mixture and the proliferation inhibitor mitomycin C showed a similar promigratory activity to that seen in wells incubated with the NT mixture alone, confirming that the restitutive effects seen were not dependant on cell proliferation (data not shown).

When HT29 cells were used, addition of AMP, CMP, or IMP alone, at 1 mg/ml, resulted in an increase in the rate of cell migration. In contrast, GMP given alone (1 mg/ml) was ineffective. However, addition of the mixture of all four NTs together (at a final total concentration of 1 mg/ml) resulted

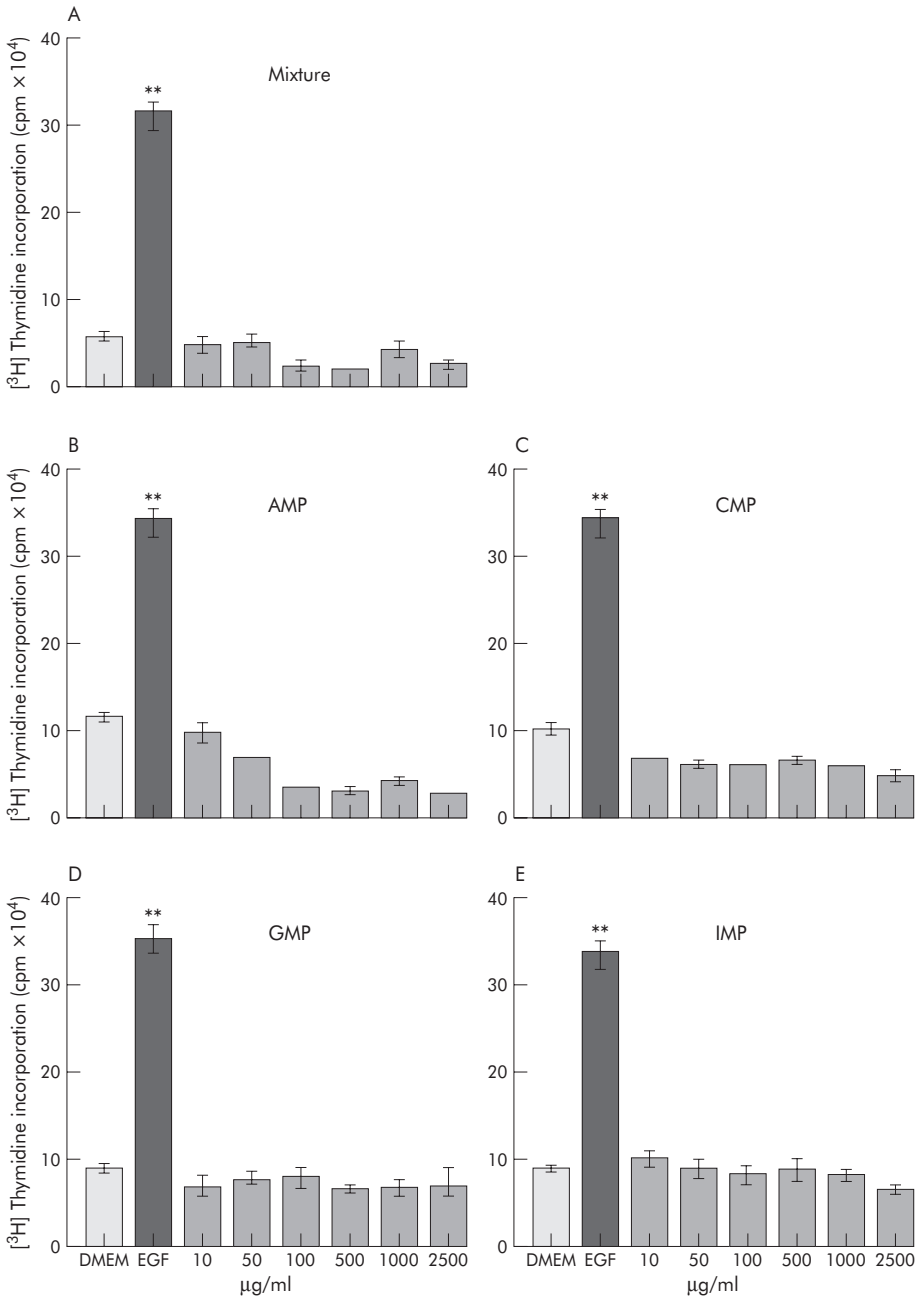

Figure 3 Effect of nucleotides (NTs) on proliferation of rat intestinal IEC6 cells. Addition of the mixture (A) or single NTs (10–2500 μg/ml) (B–E) did not stimulate proliferation compared with the negative control (Dulbecco's modified Eagle's medium (DMEM) alone). Epidermal growth factor (EGF 10 μg/ml) was used as a positive control. AMP, adenosine monophosphate; CMP, cytidine monophosphate; GMP, guanosine monophosphate; IMP, inosine monophosphate. **$p<0.01$ versus control.

in a much greater promigratory response than any of the individuals NTs given alone (fig 1C).

When IEC6 cells were used, addition of any of the single NTs alone, at 1 mg/ml, did not increase the rate of cell migration whereas a strong response was seen with the mixture (fig 1D).

Cell proliferation

In HT29 cells, addition of the mixture of NTs at 1–10 μg/ml resulted in a significant increase in thymidine incorporation. However, this effect was lost if the mixture was added at concentrations above 10 μg/ml (fig 2A).

Analyses of the effect of adding individual NTs to HT29 cells showed that the proproliferative effect was probably mainly due to the AMP constituent as AMP administered alone at 5–100 μg/ml also resulted in a dose dependant increase in proliferation (fig 2B). None of the other NTs administered alone increased proliferation in HT29 cells (fig 2C–E).

In contrast with the results seen in HT29 cells, when IEC6 cells were used, addition of the NTs, given alone or as a mixture, did not increase thymidine uptake above baseline (fig 3).

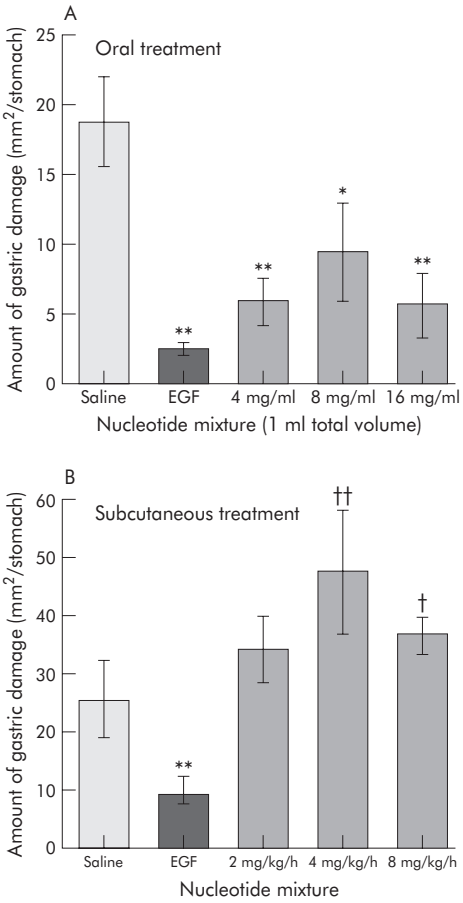

Figure 4 Effect of nucleotide (NT) administration on the amount of indomethacin induced gastric damage. (A) Rats (n = 9–16) were given an oral gavage (1 ml) of saline (negative control), epidermal growth factor (EGF 25 μg/ml, positive control), or the NT mixture (4, 8, or 16 mg/ml). All solutions also contained 2% hydroxypropylmethylcellulose to delay gastric emptying. Thirty minutes later they were placed in restraint cages, given indomethacin 20 mg/kg (subcutaneously), and left in the cages for a further three hours. At the end of the study, animals were killed and the amount of macroscopic and microscopic (data not shown) damage (per mm^2/stomach) determined. *$p<0.05$, ** $p<0.01$ versus saline control. (B) To examine the importance of the route of administration, additional rats were placed in restraint cages and a continuous subcutaneous infusion of saline (negative control) or NT mixture (2–8 mg/kg/h in saline) started. EGF (5 μg/kg/h) in saline was used as a positive control. Thirty minutes later all animals also received indomethacin 20 mg/kg (subcutaneously) and were left for a further three hours. At the end of the study, animals were killed and the amount of macroscopic and microscopic (data not shown) damage (per mm^2/stomach) determined. **$p<0.01$, less damage versus control; †$p<0.05$, ††$p<0.01$, more damage versus control.

Effect of the nucleotide mixture on an in vivo rat gastric damaging model

Oral administration of the NT mixture significantly reduced the degree of macroscopic indomethacin/restraint induced gastric damage (fig 4A). Similar results were found when the same tissue was analysed by microscopic scoring (data not shown).

In contrast with the findings of giving NT by gavage, subcutaneous administration of the NT mixture did not reduce the amount of indomethacin/restraint induced macroscopic gastric damage at any of the doses used (fig 4B) and, at the two higher doses tested (4 and 8 mg/kg/h), the amount of macroscopic damage induced was significantly increased. Assessment of the same tissues using the microscopic scoring system gave similar results (data not shown).

In both oral and subcutaneous treated animals, median intragastric pH values for animals receiving control and test factor infusions were similar, at about pH 2.2 (range 1.8–2.6).

DISCUSSION

We have shown that NTs, which are currently commercially available in health food stores in either pure or mixed forms, induce promigratory activity. They may also possess proproliferative activity, dependant on the cells being tested. In addition, we showed that a NT mixture was able to reduce the degree of injury sustained in an in vivo rat gastric damaging model.

For the in vitro studies, rat small intestinal (IEC6) and human colonic (HT29) cells were used to the examine the effects on cells from different species and regions of the bowel. We found that AMP, CMP, and IMP given alone stimulated migration of human HT29 cells whereas GMP did not. In addition, we found additive/synergistic effects when the mixture of NTs was given together. These results extend the previous observation by Dignass *et al* that addition of ATP or ADP stimulates restitution of IEC6 cells.[6] This group did not assess the other NTs and in contrast with our findings they did not find a promigratory effect using AMP. The reasons for this difference in AMP results in IEC6 cells is unclear.

In IEC6 cells, no effect on proliferation was seen when CMP, GMP, or IMP was added, whereas AMP or the NT mixture tended to reduce the degree of proliferation. Our results are generally in keeping with the findings of Dignass *et al* who reported antiproliferative effects of ATP and ADP when added to IEC6 cells.[6] Interestingly, a different set of results were seen in our studies when we also examined HT29 cells. In these cells, a proproliferative effect was seen when AMP or the NT mixture was used. These results confirm the need for caution in extrapolating results from in vitro studies, usually using cancer cell lines, to the in vivo situation. The effects of NTs on proliferation do however appear to vary with the cells under study as placental derived polydeoxyribonucleotides have been reported to increase proliferation of primary skin fibroblasts.[7] A potential explanation for these differences in results may be the presence or absence of relevant NT receptors on the cells under study. Nucleotides act on at least two families of receptors: the ionotropic P2X receptors and the G protein coupled P2Y

receptors. Whereas P2X receptors are predominantly receptors of ATP, the different P2Y receptors are activated by distinct NTs, diphosphates or triphosphates, or purines or pyrimidines, some of them being conjugated to sugars. The finding that the effects on proliferation and migration were dissociated in the two cell lines (that is, promigration was seen in both cell lines whereas different effects were seen on proliferation) suggests that the receptors involved in the two processes are likely to be different. Additional experiments, including detailed pharmacological analogue studies, are required to unravel this further. For detailed review of NT receptors see Murugappan and colleagues.[8]

Studies examining the potential beneficial effect of the NT mixture on gastric injury were performed using indomethacin induced injury in rats as we have previously validated this model for other bioactive agents such as bovine colostrum,[4] and non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, continue to be a major cause of morbidity and mortality in humans.[9] As is the situation with cell culture studies, caution always has to be shown in extrapolating results obtained from animal models to the human situation.

Results from the restitution studies suggested that additive synergistic effects were present if a mixture of NTs were used. In addition, most nutritional supplements tend to use mixtures of NTs rather than a single one. We therefore decided to examine the effects of a mixture of NTs in our in vivo study. We found that oral administration of the NT mixture was capable of reducing gastric injury by approximately 60% whereas systemic administration was unhelpful and actually increased the degree of injury. Taken together, these results suggest that the protective actions of oral NTs were mediated via local mechanisms. Importantly, the concentrations used in our restitution studies (1 mg/ml), and in our gastric damage model (4–16 mg/ml), are likely to be present in the human gastric juice of subjects taking NT supplements, as the NT components within these products usually comprise 1–100 mg/capsule, dependant on the source and individual product.

The mechanism(s) by which oral administration of NTs decreased gastric injury is unclear. This effect did not appear to be mediated through alteration in gastric acid and therefore NTs can be considered as cytoprotective agents. Indomethacin causes damage to the gastrointestinal tract by several mechanisms, including reduction of mucosal prostaglandin levels, reduction of mucosal blood flow, stimulating neutrophil activation, and possibly also stimulating apoptosis.[10] It is possible that several of these factors may have been influenced by the NTs and further work is required to understand this process. While it is possible that the gastroprotective effects seen were mediated through topical physicochemical actions, the results from our in vitro studies suggested that protective mechanisms, such as stimulation of restitution, may have been mediated through NT receptors.

Hundreds, if not thousands, of products are currently marketed as ''health food supplements''.[11] Seventy two per cent of the American population use one or more health supplement products regularly and 57% considered that these therapies reduced their need for drugs and other medical therapies. This results in an annual turnover of around £1.4 billion ($2.4 billion) in the UK and £10 billion ($18 billion) in the USA, with about 8% annual growth.[1] The sources of these ''natural'' products are diverse and include bacteria (for example, probiotics), plant (for example, prebiotics, herbal remedies), animal (for example, colostrum), insect (for example, honey), and marine (for example, sponges, snails) origins. In relation to NTs for human use, these may be obtained from bacterial cultures but also from human placentas.[7] A fuller review of the potential sources of nutriceuticals is provided by Ghosh and Playford.[1] Unfortunately, current evidence of the scientific validity of most of these traditional and commercial compounds is severely limited and the level of evidence used in support of their claims often falls well below that acceptable in the medical and scientific community.

The NTs investigated in this study are already marketed in the USA as ''over the counter'' health food supplements and, as with many of these products, the major marketing strategy is via patient validatory statements. There have however been some limited scientific studies; for example, an in vitro study found that adenine NT stimulated the rate of wound healing in monkey renal tubular epithelial cells.[12] Similarly, an NT mixture was effective when administered intravenously to rats with D-galactosamine induced liver injury.[13] In addition, small pilot human studies have shown that polydeoxyriboNT, given as eye drops, increased corneal epithelial regeneration[14] and when given by combined intramuscular and subcutaneous route, increased the rate of healing of autologous skin grafts.[15]

In conclusion, our studies have shown that NTs, commercially available as over the counter ''health food'' supplements for licensing purposes, possesses biological activity when assessed using several models of gut integrity and repair. They were able to induce the same biological repair promoting responses as the potent ''drug'' epidermal growth factor when tested under both in vitro (promigratory) and in vivo (gastric damage) conditions (albeit at different concentrations). These results further emphasise that the division between ''food products'' and ''drugs'', when considered in terms of biological activity, is far from clear, and that these products should be considered as ''nutriceutical'' or functional foods. Similarly, the idea that natural products are ''safe'' whereas conventional drugs are ''dangerous'' is a gross oversimplification. For example, although we have shown a potentially useful effect in the current series of studies with regard to oral administration of NTs and gastroprotection, systemic administration actually caused exacerbation of the degree of gastric injury. The reason(s) behind this difference is unclear and deserves further investigation. Similarly, a study in rats on the effect of dietary supplementation with a nucleoside/NT mixture showed that it exacerbated the injury associated with dextran sodium sulphate induced colitis.[16] Further studies on the potential benefits (and risks) of NT supplementation, therefore, appear justified.

ACKNOWLEDGEMENTS

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Conflict of interest: None declared.

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EDITOR'S QUIZ: GI SNAPSHOT

doi: 10.1136/gut.2005.073247

Answer

From question on page 164

The patient had dominant dorsal pancreatic duct syndrome and intraductal papillary mucinous neoplasm (IPMN) in the ventral pancreatic duct.

Figures 1 and 2 showed separated drainage of the common bile duct and pancreatic duct into the duodenum. The pancreatic duct could only be opacified by cannulation at the minor papilla, not at the major papilla. We performed endoscopic ultrasonography, which revealed echogenic nodules within the dilated ventral pancreatic duct. She received a modified Whipple operation with resection of the pancreatic head, where the anatomical abnormalities were located. Photography of the resected specimen (fig 3) demonstrated a prominent dorsal pancreatic duct (DPD) connecting to the minor papilla (mP). The ventral pancreatic duct (VPD) manifested as a torturously septated duct without grossly identifiable connection to the major papilla. Microscopically, IPMN with borderline malignancy potential was diagnosed over the ventral pancreatic duct (fig 4, haematoxylin-eosin stain, original magnification ×100). The postoperative course was uneventful and she has had regular follow ups without recurrence of pancreatitis.

In this patient, mucin producing IPMN caused pancreatic outflow obstruction and contributed to her recurrent attacks of pancreatitis. Typically, IPMN is characterised by excess mucus at the major papilla. The characteristic ''fish mouth'' appearance of the major papilla may be absent in the early stages of IPMN, as is our case. Considering the synchronous and metachronous malignancy potential in cases of IPMN, close postoperative surveillance is mandatory for these patients.

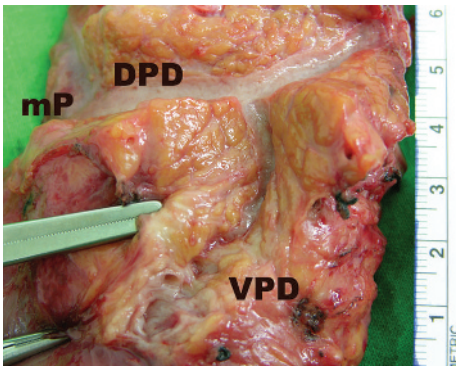

Figure 3 Photography of the dissected specimen of the pancreatic head. VPD, ventral pancreatic duct; DPD, dorsal pancreatic duct; mP, minor papilla.

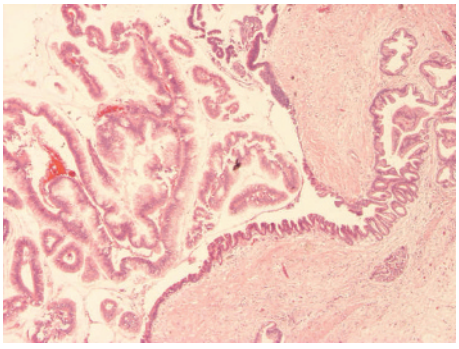

Figure 4 Microscopic examination of the dilated ventral pancreatic duct showing intraductal papillary mucinous neoplasm (haematoxylin-eosin stain, original magnification ×100).