

A Novel Adenosine Precursor 2',3'-Cyclic Adenosine Monophosphate Inhibits Formation of Post-surgical Adhesions

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Abstract

Background Intraperitoneal adenosine reduces abdominal adhesions. However, because of the ultra-short half-life and low solubility of adenosine, optimal efficacy requires multiple dosing.

Aim Here, we compared the ability of potential adenosine prodrugs to inhibit post-surgical abdominal adhesions after a single intraperitoneal dose.

Methods Abdominal adhesions were induced in mice using an electric toothbrush to damage the cecum. Also, 20 μ L of 95 % ethanol was applied to the cecum to cause chemically induced injury. After injury, mice received intraperitoneally either saline ($n = 18$) or near-solubility limit of adenosine (23 mmol/L; $n = 12$); 5'-adenosine monophosphate (75 mmol/L; $n = 11$); 3'-adenosine monophosphate (75 mmol/L; $n = 12$); 2'-adenosine monophosphate (75 mmol/L; $n = 12$); 3',5'-cyclic adenosine monophosphate (75 mmol/L; $n = 19$); or 2',3'-cyclic adenosine monophosphate (75 mmol/L; $n = 20$). After 2 weeks, adhesion formation was scored by an observer blinded to the treatments. In a second study, intraperitoneal

adenosine levels were measured using tandem mass spectrometry for 3 h after instillation of 2',3'-cyclic adenosine monophosphate (75 mmol/L) into the abdomen.

Results The order of efficacy for attenuating adhesion formation was: 2',3'-cyclic adenosine monophosphate > 3',5'-cyclic adenosine monophosphate \approx adenosine > 5'-adenosine monophosphate \approx 3'-adenosine monophosphate \approx 2'-adenosine monophosphate. The groups were compared using a one-factor analysis of variance, and the overall p value for differences between groups was $p < 0.000001$. Intraperitoneal administration of 2',3'-cAMP yielded pharmacologically relevant levels of adenosine in the abdominal cavity for >3 h.

Conclusion Administration of 2',3'-cyclic adenosine monophosphate into the surgical field is a unique, convenient and effective method of preventing post-surgical adhesions by acting as an adenosine prodrug.

Keywords Post-surgical adhesions · Adenosine · Adenosine prodrug · 2',3'-Cyclic adenosine monophosphate

Introduction

Abdominal adhesions are internal fibrous bands that form between abdominal tissues and organs and represent an exaggerated healing response by the body secondary to surgically induced trauma following open and laparoscopic procedures. Post-surgical adhesions remain a common clinical problem occurring in 93 % of patients undergoing abdominal surgery, resulting in infertility, obstruction of the small bowel and chronic pelvic pain [1, 2]. Adhesions from previous surgeries also increase the risk, difficulty and complications of subsequent surgery. Therefore, adhesions account for a large financial burden resulting in

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an approximately \$1.18 billion for inpatient care based on a survey of hospitalizations (National Hospital Discharge Survey) between 1998–2002 [1, 2].

Current therapies to prevent post-surgical abdominal adhesions are inadequate, and finding solutions remains elusive and is an important focus of health care research [1, 2]. The difficulty partially resides in understanding the exact pathogenesis of adhesion formation so that reversal of the mechanisms responsible does not impede normal healing processes or result in excessive bleeding. Although the etiology is complex, a unifying concept proposes that surgically induced trauma and microvascular ischemia evoke a robust inflammatory response leading to activation of the coagulation system with subsequent development of fibrin deposits [3–5]. Although there are numerous therapies, including solid and fluid/gel barriers, no FDA-approved device or pharmacologic agent appears to decrease the clinical consequences of adhesions [2, 6, 7]. Clearly, novel treatments for the prevention of post-surgical adhesions and tissue protection are needed, particularly those that can be easily and conveniently administered as a solution during minimally invasive surgery and are able to protect tissues both at the surgical site and throughout the abdominal cavity.

Adenosine is an endogenous nucleoside that is an agonist for four G-protein coupled receptors (A_1 , A_{2A} , A_{2B} , A_3) that reduce inflammation [8–11], particular in the intestines [12, 13], and inhibit fibroblast migration, proliferation and collagen formulation [14–18]. Previous studies from our laboratory demonstrate that adenosine significantly inhibits post-surgical adhesion formation in rodents [19] and pigs (preliminary data) following severe peritoneal abrasion. However, with adenosine per se, there are a number of practical limitations that reduce its potential efficacy as an optimal anti-adhesion agent. These include its limited solubility (approximately 23 mmol/L) and short half-life (in rats adenosine resides in the peritoneal cavity for only a few minutes after instillation), which together necessitate frequent administration of adenosine to elicit a maximal therapeutic response [19]. Although high peritoneal adenosine concentrations of adenosine do not affect other organ systems [20], it is conceivable that accelerated absorption of adenosine may occur in the setting of an inflamed peritoneum.

We hypothesize that administration of a non-cell-membrane permeable adenosine prodrug would provide more sustained levels in the abdominal cavity and would avoid potential toxicity on other organs. In this regard, our recent studies show that 2',3'-cyclic adenosine monophosphate (2',3'-cAMP), a positional isomer of 3',5'-cyclic adenosine monophosphate (3',5'-cAMP) and formed from the metabolism of mRNA, is metabolized extracellularly to adenosine by organ systems [21–30]. Because 2',

3'-cAMP is even more hydrophilic than 3',5'-cAMP [31], the absorption of 2',3'-cAMP would be limited because of lack of diffusion across cell membranes. The conversion of 2',3'-cAMP to adenosine involves first the metabolism of 2',3'-cAMP to 2'-adenosine monophosphate (2'-AMP) and 3'-adenosine monophosphate (3'-AMP) by 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase) and 2',3'-cyclic nucleotide-2'-phosphodiesterase, respectively, followed by conversion of these AMPs to adenosine. Therefore, if metabolism of 2',3'-cAMP is slow due to low levels of these enzymes in the peritoneal cavity, the anti-adhesive effects of adenosine would be enhanced because of the prolonged duration of 2',3'-cAMP in the peritoneal cavity. Also, since 2',3'-cAMP is highly hydrophilic, it is therefore highly water soluble, being stable in aqueous solutions of 75 mmol/L. Thus, high concentrations of 2',3'-cAMP can be readily instilled into the peritoneal cavity which would also prolong the duration of action. Similarly 3',5'-cAMP could also function as an adenosine prodrug since it is also water soluble and can be converted to adenosine [32–39].

The purpose of the present study was to improve upon our adenosine-based strategy for attenuating the formation of post-surgical adhesions. We therefore compared the efficacy of both cAMP compounds and their metabolites with a single dose of adenosine in a mouse model of abdominal adhesion formation (i.e., physical abrasion and chemical damage to the intestines). In this regard, we used the limit of solubility for these compounds because our main interest was whether maximal possible concentrations of the cAMPs and AMPs (75 mmol/L) were more efficacious than the maximum possible concentration of adenosine (25 mmol/L), i.e., our focus was not on potency, but rather efficacy (a clinically more important parameter). Our findings demonstrate that maximal concentrations of a one-time application of 2',3'-cAMP are more efficacious than adenosine, 3',5'-cAMP, 2'-AMP, 3'-AMP or 5'-AMP in reducing adhesion formation.

Materials and Methods

Animals

The experiments used 6-week-old C57BL/6 mice (Taconic Farms; Germantown, NY) fed Prolab RMH 3000 rodent diet (PMI Nutrition Inc.; St. Louis, MO) and allowed access to tap water. Light cycle, relative humidity and room temperature were 7:00 am to 7:00 pm, 55 % and 22 °C, respectively. The University of Pittsburgh Institutional Animal Care and Use Committee approved the study.

Drugs

All test drugs used in this study were obtained from Sigma-Aldrich (St. Louis, MO).

Surgery

Mice were fasted overnight and then anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). The abdomen was shaved, and the incision site was cleaned with betadine solution. Surgery was performed under a laminar flow hood using instruments sterilized in a glass bead sterilizer. The abdomen was opened via a midline incision of approximately 1 cm. The cecum was exposed and placed on sterile gauze. A sterile pediatric electric toothbrush was used to damage both sides of the cecum (brushing for 1.5 min on each side). In addition, 20 μ L of 95 % ethanol was applied to the cecum to cause further chemically induced tissue injury. After 30 s, ethanol was washed from the cecum with 1 mL of test solution. Next, the cecum was returned to the peritoneal cavity. Then 1 mL of test solution was placed in the peritoneal cavity, and the abdominal incision was sutured. Test solutions were applied by Mr. Gillespie who was blinded as to which test solution a given mouse received. Twenty mice were randomly assigned to the control (saline only) group and to each of the two cAMP groups; 12 mice were randomly assigned to each of the AMP groups. However, two mice in the control group, one mouse in the 5'-AMP group and one mouse in the 3',5'-cAMP group died within a few days of surgery and were therefore excluded because no adhesion scoring was possible in these mice. Mice were treated with antibiotics [penicillin benzathine (20 mg/mL, 0.05 mL injected into the thigh) and enrofloxacin (1.15 mg/mL, 0.15 mL injected subcutaneously)], placed under a warming lamp and returned to their housing once they regained consciousness.

Scoring of Adhesions

Fourteen days later, each mouse was re-anesthetized with pentobarbital, and the abdomen was opened to provide a clear view of the intestines. The severity of adhesion formation was assessed on a scale from 1 to 5, taking into account the extent of adhesion formation. Adhesions were identified visually and by noting the difficulty of separating adjacent surfaces. Adhesion scores were assigned by Mr. Gillespie, who was blinded as to the test solutions. The scoring was based on the extent of adhesion of the cecum to itself and to adjacent tissues or organs. A score of 1 was assigned to animals when there was only minimal (approximately >10 to <20 %) adhesion of the cecum to itself (that is within the cecum core) with no adhesion of the

cecum to other adjacent structures (abdominal wall, liver or intestines). A score of 2 was assigned when there was mild (approximately >20 to <30 %) adhesion of the cecum to itself or when there was minimal (approximately >10 to <20 %) adhesion of the cecum to itself but in addition minimal adhesion of the cecum to adjacent structures. A score of 3 was assigned to animals when the adhesion of the cecum to itself was moderate (approximately >30 to <50 %), in the absence or presence of minimal adhesion of the cecum to adjacent structures. A score of 4 was assigned to animals when the adhesion of the cecum to itself was severe (approximately >60 to <80 %) or when there was moderate adhesion to adjacent structures or both. A score of 5 was assigned to animals when adhesion of the cecum to itself was so extensive that it existed as one entity (complete adhesion within itself) or when there was severe adhesion of the cecum to itself but in addition severe adhesion of the cecum to adjacent structures.

Pharmacokinetic Study

Mice ($n = 4$) were anesthetized with an intraperitoneal injection of thiobutabarbital (100 mg/kg), and body temperature was monitored and maintained using a thermostatically regulated heating plate. The trachea was cannulated with PE-90, a PE-10 catheter was inserted into the jugular vein and an infusion of 2.45 % albumin in 0.9 % saline was initiated at 10 μ L/min. With the mouse in the supine position, a suture was secured into the muscle of the mid-abdominal wall and utilized to suspend the abdominal wall in the shape of a tent. A hole was carefully made at the apex of the tent, and a PE-50 catheter was positioned deep in the peritoneal cavity. One milliliter a solution of 2',3'-cAMP (75 mmol/L) was instilled into the peritoneal cavity. Next, utilizing a new PE-50 catheter for each sample, 0.1 mL of fluid was withdrawn at 1, 15, 30, 60, 120 and 180 min following instillation and immediately placed on ice. 2',3'-cAMP, 2'-AMP, 3'-AMP, adenosine, inosine and hypoxanthine concentrations were measured with high-performance liquid chromatography–tandem mass spectrometry using a triple quadrupole mass spectrometer (TSQ Quantum-Ultra; Thermo Fisher Scientific, Waltham, MA) as previously described [27].

Statistical Analysis

Adhesion scores passed all tests for normality and equality of variances. Accordingly, the data were analyzed with a one-way analysis of variance, followed by a protected Fisher's least significant difference multiple-comparisons test if the overall analysis of variance was significant. Statistical analyses were conducted with the NCSS software (version 6.0; Kaysville, UT).

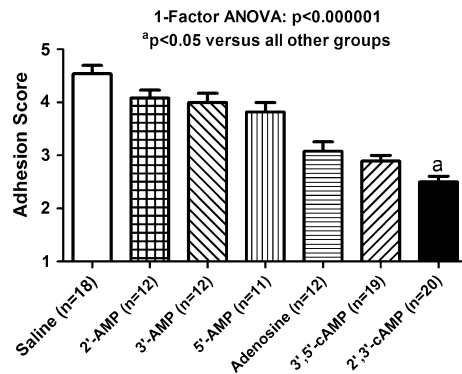


Fig. 1 Bar graph shows the adhesion scores of mice treated with a single intraperitoneal dose of the indicated treatment. Each compound was administered into the peritoneal cavity at a concentration that was approximately the limit of solubility for that compound (23 mmol/L for adenosine and 75 mmol/L for all other purines). Values are means and SEMs

Results

Six groups were treated with test solutions containing chemical agents (purines) of interest. Each compound was administered into the peritoneal cavity at a concentration that was approximately the limit of solubility for that compound (23 mmol/L for adenosine and 75 mmol/L for all other purines.) In addition, there was a seventh group (control group) in which the test solution was only the vehicle (0.9 % saline) in which the chemical agents of interest were dissolved. The order of efficacy for attenuating adhesion formation was as follows (Fig. 1): 2',3'-AMP (adhesion score of 2.5 ± 0.11) which was better than 3',5'-cAMP (adhesion score of 2.89 ± 0.11) which was approximately the same as adenosine (adhesion score of 3.08 ± 0.18) which was better than 5'-AMP (adhesion score of 3.82 ± 0.18) which was approximately the same as 3'-AMP (adhesion score of 4.00 ± 0.17) which was approximately the same as 2'-AMP (adhesion score of 4.08 ± 0.15). The seven groups were compared using a one-factor analysis of variance with independent groups. The overall p value for differences between the groups was $p < 0.000001$. Post-hoc analysis with a protected Fisher's least significant difference multiple-comparisons test was used to identify which groups were different. All treatment groups had adhesions scores that were significantly ($p < 0.05$) less than (better than) the saline control group (adhesion score of 4.54 ± 0.16). However, the adhesion scores for 5'-AMP, 3'-AMP and 2'-AMP were nominally similar, not significantly different from each other and only slightly less than (better than) that for the saline control group. This indicates that although these three adenosine monophosphates decreased adhesion formation, the effect was small. The adhesion scores for

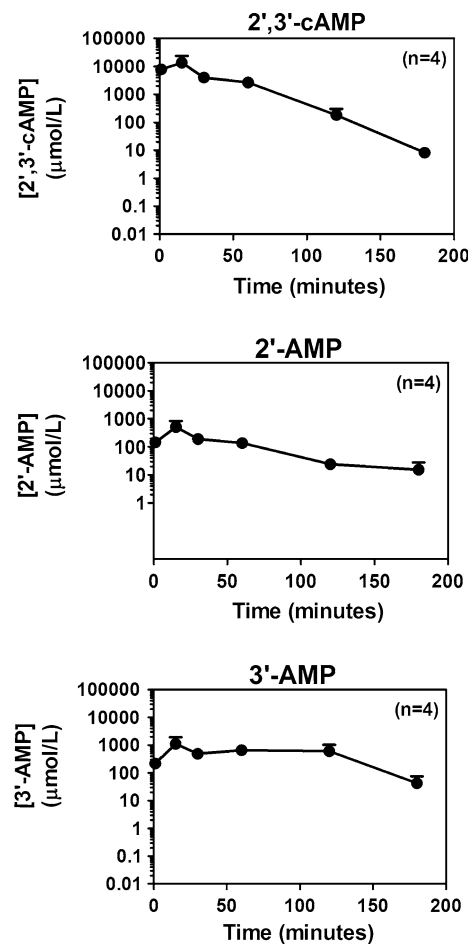


Fig. 2 Line graphs illustrate the relationship between time (1–180 min) after administration into the peritoneal cavity of 2',3'-cAMP (1 mL of a 75 mmol/L solution) and concentrations of 2',3'-cAMP, 2'-AMP and 3'-AMP. Values are means and SEMs

adenosine and 3',5'-cAMP were nominally similar, not significantly different from each other, less than (better than) 5'-AMP ($p < 0.05$), 3'-AMP ($p < 0.05$) and 2'-AMP ($p < 0.05$), but more than (not as good as) 2',3'-cAMP ($p < 0.05$). Finally, the analysis showed that 2',3'-cAMP significantly ($p < 0.05$) improved adhesion scores better than all other treatments. Although not formally scored, it was also observed that the structures within the abdominal cavity in the group treated with 2',3'-cAMP and 3',5'-cAMP appeared less inflamed and generally healthier than the other groups.

The metabolism of 2',3'-cAMP in the peritoneal cavity is shown in Figs. 2 and 3. Although concentrations of 2',3'-cAMP dropped rapidly after administration, because of the high starting concentrations, 3 h after the administration of 2',3'-cAMP concentrations of 2',3'-cAMP in the peritoneal cavity were still substantial (i.e., approximately $10 \mu\text{mol/L}$; Fig. 2, top panel). Within a minute of application of 2',3'-cAMP, levels of 2'-AMP (Fig. 2, middle panel) and

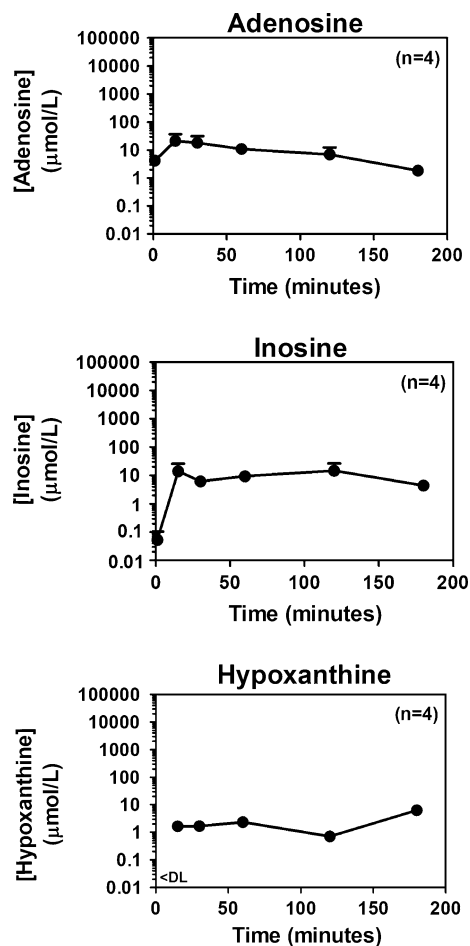


Fig. 3 Line graphs illustrate the relationship between time (1–180 min) after administration into the peritoneal cavity of 2',3'-cAMP (1 mL of a 75 mmol/L solution) and concentrations of adenosine, inosine and hypoxanthine. The level of hypoxanthine at 1-min post-administration was below the assay's detection limit (<DL). Values are means and SEMs

3'-AMP (Fig. 2, bottom panel) achieved concentrations of >100 μmol/L, which were relatively maintained for 2 h, and even at 3 h post administration of 2',3'-cAMP, levels of 2'-AMP and 3'-AMP remained above 10 μmol/L. More importantly, within 1 min after administering 2',3'-cAMP, adenosine levels in the peritoneal cavity exceeded 1 μmol/L (Fig. 3, top panel) and quickly rose to above 10 μmol/L, and even at 3 h, adenosine levels in the peritoneal cavity were pharmacological significant (>1 μmol/L, which is a concentration that activates adenosine receptors [40]). One minute after administration of 2',3'-cAMP, levels of inosine (Fig. 3, middle panel) were extremely low (<0.1 μmol/L), and levels of hypoxanthine (Fig. 3, bottom panel) were below detection limit. However, within 15 min, inosine levels increased to >10 μmol/L, and hypoxanthine concentrations increased to >1 μmol/L. Both inosine and hypoxanthine remained increased for >3 h.

Discussion

The most striking finding of this study is that a single intraperitoneal dose of 2',3'-cAMP is highly effective in attenuating adhesion formation in a small animal model subjected to extreme tissue injury (3 min of brushing the cecum with an electric toothbrush plus chemical injury with ethanol). This study also confirms prior results from our group that a single adenosine dose is anti-adhesive [19]; however, the present results show that 2',3'-cAMP is more efficacious than adenosine per se. Although a lower concentration of adenosine was used compared to 2',3'-cAMP, this is a valid comparison for clinical efficacy since higher concentrations of adenosine cannot be dissolved in saline; therefore, an important advantage of 2',3'-cAMP is its higher aqueous solubility, thus yielding a higher achievable (in practice) efficacy. We conclude that 2',3'-cAMP may be a preferred therapy for reducing the formation of post-surgical adhesions.

Adenosine activates A₁, A_{2A}, A_{2B} and A₃ receptors, and inosine is a partial agonist at A₁ and A₃ receptors [40]. The present study shows that a one-time intraperitoneal dose of 2',3'-cAMP utilized for adhesion studies results in concentrations of adenosine and inosine in the abdominal cavity of >1 μmol/L for >3 h. These are pharmacologically active concentrations of adenosine and inosine [40]. Indeed, because adenosine can bind to A₁ and A_{2A} receptors at low nmol/L concentrations [40], it is likely that pharmacologically active levels of adenosine are achieved for much longer than 3 h. Therefore, it is likely that 2',3'-cAMP functions as an adenosine prodrug to provide prolonged levels of adenosine (and inosine) in the peritoneal cavity, thus avoiding the necessity of multiple applications of adenosine to optimize adenosine's anti-adhesive effects as previously demonstrated in the rat model [19].

Our recently published *in vivo* studies demonstrate the important role of CNPase in the conversion of 2',3'-cAMP to adenosine [29]. Since CNPase is a rate-limiting enzyme for the metabolism of 2',3'-cAMP to adenosine, we speculate that low CNPase levels may be present in the abdomen, which serves to slow the metabolism of 2',3'-cAMP and prolong its duration of action. Indeed, our preliminary studies in rats confirm that protein levels of CNPase are low in the peritoneum and small intestines compared with the brain. Therefore, 2',3'-cAMP may be an ideal slow-drug release technology because it is slowly metabolized extracellularly in the abdomen to adenosine, a potent anti-adhesive agent.

The marked beneficial effect of 2',3'-cAMP in preventing abdominal adhesions is most likely secondary to increased levels of adenosine in the peritoneal cavity for hours following injury. While the pathogenesis of adhesions is complex and multifactorial, adenosine via activation of

numerous extracellular receptors modifies a number of the proposed cellular and biochemical pathways involved [19]. Adenosine would reverse the initial event of microvascular ischemia induced by surgical trauma via vasodilatation through activation of adenosine receptors. Indeed, our recently published studies show that *in vivo* 2',3'-cAMP causes vasodilatation via activation of adenosine receptors [30]. In addition to vasodilatation, 2',3'-cAMP, via adenosine, stimulates the proliferation of vascular endothelial cells, which would accelerate vascular repair [41]. Moreover, 2',3'-cAMP, via adenosine, enhances the proliferation of epithelial cells [41], which would hasten re-epithelialization of the mesothelial surface. Although the effects of 2',3'-cAMP on the immune system are unknown, adenosine is well known to reduce the secondary inflammatory response to tissue injury via activation of adenosine receptors on various inflammatory cells including neutrophils, lymphocytes and macrophages [9, 11, 12]. It is also well accepted that adenosine inhibits release of cytotoxic oxygen free radicals and proteolytic enzymes from neutrophils, reduces lymphocyte proliferation and increases suppressor subtypes and inhibits macrophage production of various inflammatory cytokines—such as TNF α and interleukins 6 and 8 [9, 11, 12]. Finally, studies show that adenosine would also modify the final stage of adhesion formation due to inhibition of fibroblast proliferation via activation of A_{2B} receptors [14–18].

Why was 2',3'-cAMP more efficacious than 2'-AMP and 3'-AMP even though 2',3'-cAMP is metabolized to adenosine via the intermediate AMPs (i.e., 2'-AMP and 3'-AMP)? It is possible that very high concentrations of these AMPs are pro-inflammatory and cause vasoconstriction. In this regard, our preliminary (unpublished) data in human CD4+ T cells suggest that very high concentrations of 2'-AMP stimulate TNF α production. Also, our preliminary (unpublished) data in the rat kidney indicate that high concentrations of 2'-AMP and 3'-AMP can cause vasoconstriction. Therefore, when using mM concentrations of 2'-AMP and 3'-AMP, the direct pro-inflammatory and vasoconstrictor effects of 2'-AMP and 3'-AMP likely attenuate any protection by generated adenosine. In contrast, when 2',3'-cAMP is administered, 2',3'-cAMP acts as a “reservoir” of adenosine yet generates much lower concentrations of the intermediate AMPs (too low to cause adverse effects).

As recently reviewed by Eltzschig, Sitkovsky and Robson [42], adenosine signaling affords, via A_{2A} and A_{2B} receptors, both anti-inflammatory and barrier-protective actions during intestinal inflammation. Importantly, inflammation and hypoxia, via hypoxia-dependent transcription factors, upregulate A_{2A} and A_{2B} receptors, increase the expression of CD39 (metabolizes ATP to ADP and 5'-AMP) and CD73 (metabolizes 5'-AMP to

adenosine) and decrease the expression of equilibrative nucleoside transporters [43]. These concepts suggest that endogenous adenosine may importantly protect against post-surgical adhesions because surgery no doubt induces inflammation, which would activate the aforementioned mechanisms that accelerate adenosine formation and slow adenosine removal from the extracellular compartment. By analogy, it is conceivable that the enzymes involved in 2',3'-cAMP metabolism to adenosine are also up-regulated by intestinal injury, hypoxia and inflammation, which would make 2',3'-cAMP an even more effective in combating the formation of post-surgical adhesions.

Currently approved therapies for prevention of adhesions in humans are based on application of liquid or solid barriers to physically separate damaged tissue. These devices have a number of limitations and do not appear to prevent small bowel obstruction [1, 6]. Therefore, there is a dire need to develop new, safe and easily administered drug technologies to prevent adhesions following abdominal surgical procedures [4]. There are numerous reports of drug therapies, tested in animals, targeting specific pathways in adhesion formation [1, 2, 4, 44, 45]. Current consensus demonstrates that these agents have limited success and have significant adverse effects. Following the discovery of 2',3'-cAMP, we postulated that 2',3'-cAMP would be a promising anti-adhesive agents for the following reasons: First, 2',3'-cAMP would function as an adenosine prodrug since it is metabolized to adenosine. Second, because the metabolism of 2',3'-cAMP could be slow in tissues expressing only low levels of CNPase, 2',3'-cAMP could result in significant and prolonged levels of adenosine in the peritoneal cavity and hence would require only a one-time administration. Third, adenosine per se is likely superior to other pharmacological agents since adenosine modulates numerous pathways postulated to produce adhesions. Fourth, 2',3'-cAMP is cheap and highly water soluble allowing for instillation of high concentrations into the abdominal cavity. Fifth, the hydrophilicity of 2',3'-cAMP and its slow metabolism to adenosine would avoid rapid absorption of 2',3'-cAMP or adenosine, thus limiting adverse effects on other organ systems.

In summary, single administration of 2',3'-cAMP markedly inhibits adhesion formation in a mouse model of extreme tissue injury. Although not formally scored, structures in the abdominal cavity appeared less inflamed and healthier in animals treated with 2',3'-cAMP. Biochemical studies showed that 2',3'-cAMP results in a significant elevation of intraperitoneal adenosine for >3 h. Further studies are warranted to examine the potential of this inexpensive, water soluble, and easily formulated compound to inhibit surgical adhesions in large animal models.

Conflict of interest The University of Pittsburgh is the applicant of a pending patent application entitled “Cyclic Adenosine Monophosphates for Reducing the Formation of Adhesions”, and E. K. Jackson is listed as the inventor on this patent application.

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