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Synthesis and Pharmacological Screening of Pyridopyrimidines as Effective Anti-Diarrheal Agents through the Suppression of Cyclic Nucleotide Accumulation

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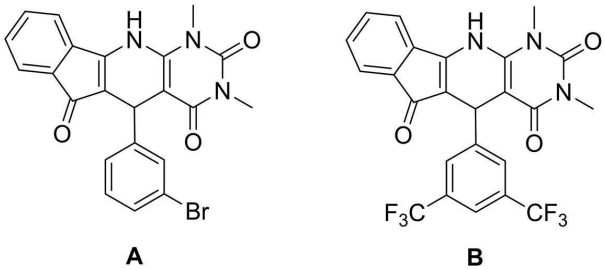
The increased levels of cyclic nucleotides (cGMP and cAMP) in enterocytes trigger intracellular mechanisms of ion and fluid secretion into the lumen, causing secretory diarrhea. Twelve novel pyridopyrimidines derived from 5-(3,5-bistrifluoromethylphenyl)-1,3-dimethyl-5,11-dihydro-1H-indeno[2,1:5,6]pyrido [2,3-d]pyrimidine-2,4,6-trione (FPIPP) were synthesized and evaluated on intracellular cyclic nucleotide accumulation. All compounds had no effect on either cyclic nucleotide basal levels or on pre-contracted aortic rings. The metabolic activity and viability in T84 cells, assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and the LDH (lactate dehydrogenase) assays, respectively, were not affected by incubation with the compounds (50 μM). Compound **VI** almost abolished cGMP accumulation (94% inhibition) induced by STa toxin in T834 cells and significantly reduced (69%) forskolin-induced cAMP accumulation in Jurkat cells. Compound **VI** was active in an in vivo model for diarrhea in rabbits. These results prompted us to perform a microscopic histopathological analysis of intestinal tissues, showing that only compound **VI** preserves the intestine without significant pathological changes and with a decreased inflammatory pattern in comparison to FPIPP. In vitro stability test revealed that compound **VI** is resistant to oxidation promoted by atmospheric oxygen.

1. Introduction

Diarrhea is a major health issue, especially in developing countries where it is estimated to be the second cause of death in children under five years of age, killing more children than AIDS, malaria and measles combined.[1–4] Almost 60% of deaths associated with diarrhea are linked to enterotoxigenic strains of bacteria, such as *Escherichia coli* (from here *E. coli*).[5] These strains are able to interact with host epithelial cells through specific adhesins, colonizing the small intestine. These strains are able to synthesize numerous toxins, like enterotoxin "A" thermostable (STa). STa binds to the membrane receptor guanylate cyclase type C (GC-C) in intestinal epithelial cells, stimulating the synthesis of 3′-5′ cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP).[6–9] STa toxin perturbs electrolyte homeostasis in intestinal cells determining secretory diarrhea that requires immediate rehydration. *E. coli* strains are responsible of about 400 million cases of diarrhea in 5 or less years old children worldwide.[10]

The 5-(3-bromophenyl)-1,3-dimethyl-5,11-dihydro-1H-indeno-[20:10:5, 6]pyrido[2,3-d] pyrimidine-2,3,6-trione (BPIPP, compound **A**, Figure 1) and some other related compounds, like the 5-(3,5-bistrifluoromethylphenyl)-1,3-dimethyl-5,11-dihydro-1H-indeno[2,1:5,6]pyrido[2,3-d]pyrimidine-2,4,6-trione (FPIPP, compound **B**, Figure 1) suppress the synthesis of cGMP when in presence of STa and other toxins in vitro.[11,12]

Here it is reported the design, synthesis and the pharmacological in vitro effect of twelve novel pyridopyrimidine derivatives (**I**–**XII**, Table 1) on cyclic nucleotides accumulation. The



A B

Figure 1. Chemical structures of BPIPP (**A**) and FPIPP (**B**).

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Table 1. Conventional heating versus microwave irradiation in the synthesis of aldehydes **3b** and **3c** and of pyridopyrimidines **I–XII**.

	Conventional heating (oil bath)			Microwave irradiation			
Compd	Time [h]	Temp [°C]	Yield[a] [%]	Power	Time [min]	Temp [°C]	Yield[a] [%]
3b	12	70	88	200	120	70	95
3c	12	70	85	200	120	70	94
I	8	reflux	52	400	40	120	91
II	8	reflux	38	400	40	120	75
III	8	reflux	28	400	40	120	67
IV	8	reflux	28	400	40	120	64
V	8	reflux	30	400	40	120	72
VI	8	reflux	38	400	40	120	85
VII	8	reflux	46	400	40	120	90
VIII	8	reflux	35	400	40	120	78
IX	8	reflux	27	400	40	120	73
X	8	reflux	37	400	40	120	80
XI	8	reflux	75	400	40	120	94
XII	8	reflux	70	400	40	120	96

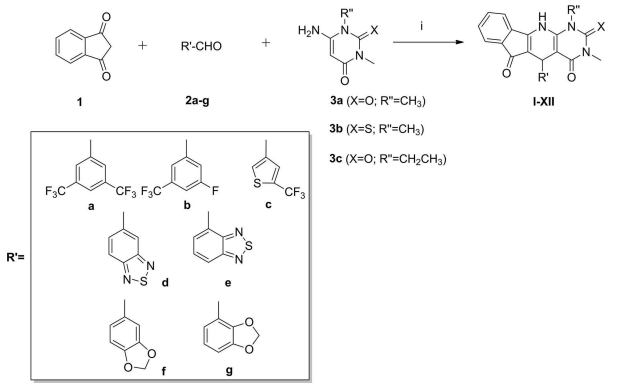
[a] All the reactions were performed three times and the reaction time and yields given are the average values.

most potent compound (**VI**) was further evaluated in an in vivo model of diarrhea in rabbits and an histopathological analysis of intestinal tissue treated with this compound was performed by microscopic analysis. Moreover, bearing in mind that dihydropyridines are reported in literature to be converted by air oxygen into their corresponding pyridine analogues, the chemical stability compound **VI** toward air oxidation was also assessed both in solid state and in methyl alcohol solution.

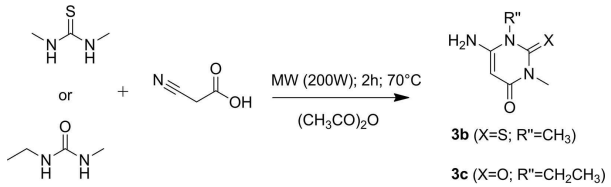
2. Results and Discussion

2.1. Synthesis of Pyridopyrimidine Derivatives

Compounds **I–XII** were prepared following, with modifications, the synthetic procedures[13] depicted in Scheme 1. The synthetic procedure is based on the Hantzsch dihydropyridine three component cyclization.[14,15] In particular, reaction of 1,3-indandione (**1**) with the aldehydes (**2a–g**) and the opportune primary amines (**3a–c**) in acetic acid afforded the desired compounds **I–XII**. Aldehydes **2a–2c** were commercially available, while **2d–2g** were synthesized as previously described.[16–19] Intermediate **3a** was commercially available, while **3b** and **3c** were prepared according to that reported in Scheme 2 following, with modifications, procedures previously reported in literature.[20]



Scheme 1. General procedure for the synthesis of compounds **I–XII**: i) CH_3COOH, sealed vessel, 120 °C, MW (400 W) 40 min.



Scheme 2. Synthetic route for intermediates **3b** and **3c**.

Conventional heating (oil bath) and microwave irradiation of the reactions were compared (Table 1).

The main advantage of the microwave procedures was that a short time of irradiation of the reaction mixtures provided the derivatives **I–XII** and intermediates **3b** and **3c** as the major product: when the reactions were performed under controlled microwave irradiation conditions the reaction times were greatly reduced from 12 h to 2 h (intermediates **3b** and **3c**) and from 8 h to 40 min for final compounds **I–XII**.

Furthermore, in all the reactions we observed a significative increase of the obtained yield that ranged from 64 to 96% (higher in comparison to 27–70% of conventional heating).

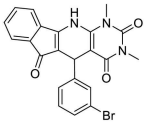
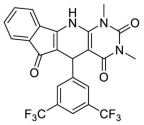
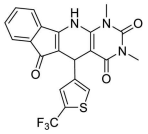
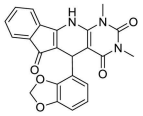
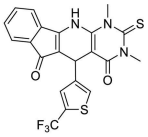
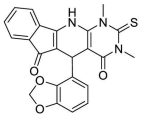
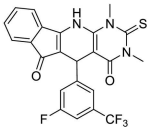
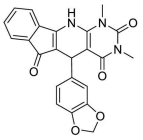
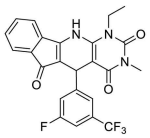
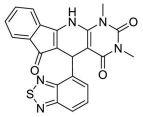
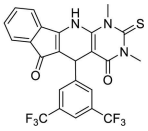
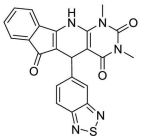
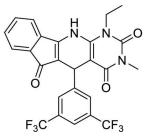
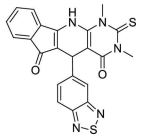
2.2. Evaluation of Pyridopyrimidine Derivatives I–XII as cGMP and cAMP Synthesis Inhibitors

The synthesized compounds were tested in vitro in order to evaluate their effects on cyclic nucleotide accumulation. Compound activity was determined measuring the concentration of cGMP or cAMP before and after stimulation with STa or Forskolin, respectively, by ELISA assay. Results obtained for cGMP and cAMP accumulation are reported in Table 2.

The basal levels of cAMP in Jurkat cells were 16 ± 1 pmol/10 min/mg and 140 ± 8 pmol/10 min/mg following stimulation by Forskolin (100 μM). The compounds had no effect on the basal intracellular cAMP levels in T84 cells (data not shown). All compounds significantly inhibited cAMP accumulation-induced by forskolin (Table 2). In the absence of STa stimulation the cGMP levels in T84 cells were 7 ± 1 pmol/10 min/mg. When stimulated by STa (1 μM) these levels enhanced to 471 ± 11 pmol/10 min/mg. None of the compounds induced changes in intracellular cGMP levels compared to baseline group (DPBS) in the absence of stimulation by STa (data not shown). With the exception of compound **VII**, all compounds inhibited cGMP accumulation-induced by STa (Table 1). A full concentration-dependent curve (Figure 2) was performed for the most active compounds (**I**, **II**, **IV**, **VI** and **XI**).

The results in cyclic nucleotides accumulation allowed some preliminary considerations of structure-activity relationships (SAR). An electronegative substituent at *meta* position of the phenyl ring (carbon 5) was essential to compound activity.

Table 2. Chemical structures of pyridopyrimidine derivatives (**I**–**XII**) and their effects on cGMP accumulation induced by STa in T84 cells and cAMP accumulation induced by Forskolin in Jurkat cells.[a]

Compd	Structure	cGMP [%]	cAMP [%]	Compd	Structure	cGMP [%]	cAMP [%]
A (BPIPP)		86 ± 4	n.a.	B (FPIPP)		93 ± 4	n.a.
I		69 ± 3	84 ± 3	VII		17 ± 7	37 ± 4
II		70 ± 5	82 ± 3	VIII		49 ± 4	45 ± 6
III		51 ± 3	88 ± 3	IX		25 ± 9	35 ± 6
IV		78 ± 4	86 ± 3	X		33 ± 8	45 ± 4
V		51 ± 4	86 ± 3	XI		71 ± 4	45 ± 2
VI		94 ± 2	69 ± 2	XII		65 ± 4	80 ± 2

[a] T84 cells were pretreated for 10 minutes with compounds at 50 μM or vehicle (0.1% DMSO) and then treated with STa (1 μM). Jurkat cells were pretreated for 10 minutes with compounds at 50 μM or vehicle (0.1% DMSO) and then treated with Forskolin (100 μM) and then intracellular cGMP or cAMP accumulation were measured. Data are mean ± SEM of 3–5 independent experiments assayed in duplicate

When this feature was not present, a severe decrease in the activity was noted (compounds **VII**–**X**), both for cGMP and for cAMP inhibition. Moreover, the presence of bicyclic aromatic groups at position 5 (compounds **VII**–**XII**) caused loss of activity when compared to the reference compounds. The replacement of the methyl group with a more steric hindered ethyl group on N1 enhanced the activity (compounds **IV** and **VI**). These considerations are in accordance with those already published in literature for analogue derivatives.[12]

The isosteric replacement of an oxygen with a sulfur atom in position 2 proved to be not directly linked to the inhibitory activity. Indeed, when this chemical modification was included it generated compounds with an overlapping activity (i.e. compounds **I**/**II** and **XI**/**XII**), or with better inhibitory effect for the compound bearing the sulfur atom (i.e. **VIII** > **VII**).

Finally compound **VI**, showing the highest effect on cGMP accumulation, has a CLogP of 5.11. This value is the highest of the series even when compared to FPIPP, that shows a cLogP of 4.58.

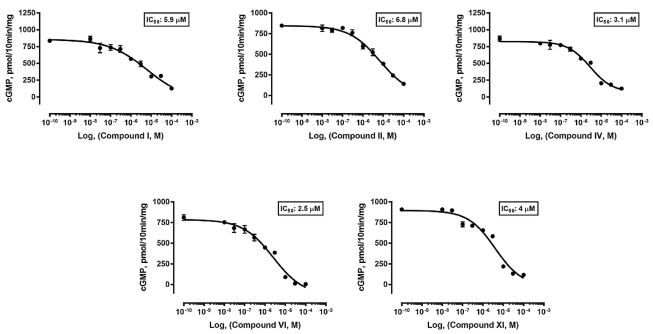


Figure 2. Concentration response curves of compounds **I**, **II**, **IV**, **VI** and **XI** (higher cGMP inhibition rate on T84 cells). Results are presented as mean ± SEM. $n = 3$–5.

On the basis of the obtained results, a pharmacological mechanism of action of the synthesized compounds has been preliminary postulated. In fact, when the STa or endogenous mediators (guanylin and uroguanylin) interact with the receptor GC-C inducing a conformational change in the extracellular portion of the homotrimeric GC-C complex, two intracellular catalytic domains dimerize and form two active catalytic clefts. This mechanism of activation in the intestine results in stimulation of Cl^- and HCO_3^- secretion (by opening of the apical CFTR Cl^- channels) and inhibition of Na^+ absorption (by blocking of the Na^+/H^+ exchanger).[7] Thus, the observed decrease in the cGMP synthesis may be related to any of the following events: (i) desensitization/allosteric modulation of the GC-C receptor or (ii) modulation of PDE5 activity.

Prolonged exposure of T84 cells to STa induces intracellular mechanisms that promote the GC-C receptor desensitization, resulting in a significant blockade in the cGMP synthesis.[21] Several compounds have been described as inhibitors of GC-C activity, even when exposed for shorter periods, similar and lower than that employed in our protocols.[22–26] Thus, it would be possible that the compounds herein described could interact and stimulate these mechanisms, responsible for the receptor desensitization, especially when highly stimulated by STa. Furthermore, it is known that when STa activates GC-C, the substrate (GMP) binds first to the regulatory site of the receptor and while this binding is required for the subsequent binding of substrate to the catalytic site, it does not affect the affinity of the catalytic site.[22] Thereby, the binding of these compounds to the regulatory site of GC-C, similarly to what can be observed for other compounds,[25–27] could inhibit its activity.

PDE5 that is highly expressed in T84 cells[28] is characterized by a relative specificity for cGMP hydrolysis at low substrate levels and by the presence of high affinity-binding sites for cGMP.[29] These binding sites are now known to be on the N-terminal regulatory GAF domains of the enzyme. The binding of cGMP to the PDE5 GAF-A domain stimulates the enzyme activity 9- to 11-fold and the blockade of this binding inhibits PDE5 activity.[29] Moreover, when cGMP levels are high (like after stimulus by STa) and GAF-A is already occupied by cGMP, PKA can also phosphorylate this site,[29] stabilizing the increased catalytic activity by enhancing the affinity of cGMP binding to the GAF-A domain.[30] However, when we incubated our compounds in T84 cells, previously treated with 3-isobutyl-1-methylxanthine (IBMX), a non-specific PDE inhibitor, the compounds still were able to block the cGMP accumulation, similarly to results described for BPIPP.[11]

2.3. Effect in Rabbit Aortic Rings

Functional studies in rabbit isolated aorta were performed to evaluate smooth muscle relaxation induced by compounds **I–XII**. Although all the compounds synthesized in this study were able to block the accumulation of cGMP and cAMP when stimulated by toxin or pharmacological agents, none of them induced changes in the basal levels of these cyclic nucleotides. This feature is extremely important, considering that small changes on cAMP or cGMP levels can promote significant physiological effects due to the importance of these intracellular signaling pathways, especially in the cardiovascular system.[31,32] Furthermore, it should be noted here the partial structural similarity of our compounds with dihydropyridine type calcium blockers, such as amlodipine, widely used in clinic as a tool to control blood pressure. These dihydropyridine derivatives act inhibiting the calcium influx through 'slow' channels in vascular peripheral and coronary smooth muscle cells, producing marked vasodilation in peripheral and coronary vascular beds.[33] Thus, the evaluation of the here described compounds effect on vascular tone is of fundamental importance. When incubated in T84 cells, amlodipine, nifedipine and nimodipine induced no changes in cGMP accumulation induced by STa (319 ± 11, 328 ± 29 and 273 ± 11 pmol/10 min/mg respectively, compared to STa 348 ± 20 pmol/10 min/mg). For this purpose, compounds **I–XII** have been evaluated in an aortic tension model, were none of them induced changes in the vascular tone, in contrast to the maximum vascular relaxation induced by amlodipine (Figure 3). Interestingly, only FPIPP

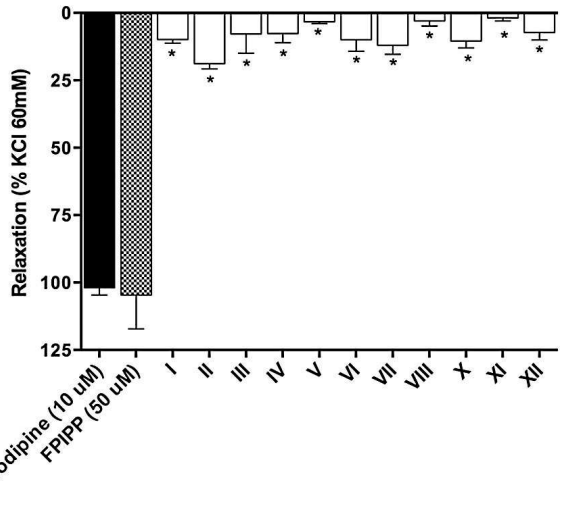


Figure 3. Effect of compounds **I–XII** on pre-contracted rabbit aortic rings. Rabbit aortic rings were pre-contracted with KCl (60 mM) and treated with 50 μM of compounds **I–XII**, FPIPP or Amlodipine (10 μM) for 40 minutes. Results are presented as mean ± SEM. $n = 3–5$. One-Way ANOVA followed by Tukey's test; *$p < 0.05$ compared to Amlodipine group.

(50 μM) promoted a dual effect by increasing the vascular tone in 20% prior induce a maximal vascular relaxation similar to amlodipine (10 μM). These observations are critically important since, despite the structural similarity between our compounds and dihydropyridine type calcium blockers, their effect on vascular smooth muscle has no physiological relevance. Moreover, the inhibition of the accumulation of cyclic nucleotides by the compounds seem to play no role in the vascular tonus, which favors the absence of possible adverse cardiovascular effects if systemic administrated.

2.4. Cell Metabolic Activity (MTT) and Cytotoxicity (LDH)

All the compounds evaluated in this study showed some effect on stimulated synthesis of cGMP and cAMP. Therefore, in order to investigate the possibility of our results be due not to an intrinsic activity of each compound, but by a toxic effect on cells, like metabolic alterations or loss of viability, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and the LDH (Lactate dehydrogenase) assays were employed.

The first assay requires cells that are actively able to metabolize MTT to an insoluble formazan precipitate.[34] To investigate changes in the metabolic activity, all the compounds were incubated for 1 hour, 24 hours and 48 hours in protein free medium. Results evidenced that no changes in cellular metabolism occurred at all tested times, suggesting the complete absence of interference on the metabolic activity of T84 cells and excluding the possibility of a metabolic interference in cells during the assay of cGMP stimulation (Table 3).

Table 3. Effect of compounds on cell metabolic activity (MTT assay).[a]

Treatment	1 h Mean [%]	S.E.M	24 h Mean [%]	S.E.M	48 h Mean [%]	S.E.M
Vehicle	100.0	–	100.0	–	100.0	–
Triton (0.1 %)	54.9*	1.1	45.2*	1.2	38.4*	1.3
STa (1 μM)	96.8	1.2	94.9	1.0	90.5	1.2
FPIPP (50 μM)	98.2	1.3	96.4	1.1	93.3	1.9
I	91.8	1.1	85.7	2.6	84.5	5.5
II	90.8	2.4	95.7	2.3	96.0	3.4
III	101.1	1.6	93.2	3.7	86.2	3.4
IV	99.3	2.5	94.4	1.6	93.4	5.1
V	101.3	3.3	92.1	4.0	88.8	3.7
VI	96.2	2.2	95.2	1.8	91.9	3.0
VII	91.7	1.1	90.1	2.7	89.4	3.9
VIII	90.5	1.4	93.8	2.6	89.6	1.6
IX	90.6	1.4	94.1	2.7	95.2	4.7
X	91.4	3.7	96.6	3.8	92.8	3.5
XI	92.7	1.0	94.6	3.3	90.6	4.0
XII	90.6	1.6	96.0	1.8	86.5	2.4

[a] T84 cells were treated with 50 μM of compounds **I**–**XII** or vehicle (DPBS with 0.1 % DMSO) for 1, 24 or 48 hours. Results are normalized to the vehicle group. $n = 3$; One-Way ANOVA followed by Tukey's test; *$p < 0.05$ compared to vehicle group.

Finally, complementary assays were performed in order to investigate cytotoxicity induced by the compounds. Intracellular enzymes are usually released after damage to the cell membrane, thus, the larger the rate of release of enzyme, the greater the extent of cell death/damage which occurred in that period.[35] In order to investigate, we measured the LDH levels in T84 cells incubated with compounds for 1 hour, 24 hours and 48 hours. Also in this case results (Table 4) were encouraging and evidenced complete absence of cytotoxicity for all the compounds at 1 h; when analyzing data at 24 h and 48 h we noticed that all compounds evidenced a very low toxic effect at 24 h with exception of compounds **III** and **V** (7.8 % and 8.6 %, respectively), while a general increasing toxicity trend at 48 h was noticed. In this latter case all the compounds, as expected, showed effects ranging between 9.2 % and 13.5 %, that are in line with those of nontoxic compounds and are moreover similar to the results obtained with BPIPP.[11]

Table 4. Effect of compounds on cell LDH release (LDH cytotoxicity assay).

Treatment	1 h Mean [%]	S.E.M	24 h Mean [%]	S.E.M	48 h Mean [%]	S.E.M
Vehicle (DMSO 0.1 %)	1.9	1.0	1.6	0.5	2.2	0.4
Positive control (LDH)	61.4 %*	1.5	65.8*	3.9	65.5*	2.7
STa (1 μM)	2.3	0.5	2.8	0.4	3.7	1.1
FPIPP (50 μM)	2.9	0.3	4.2	0.8	9.3	1.5
I	2.1	0.2	2.7	1.1	12.5*	0.7
II	2.2	0.5	6.1	1.5	9.2	1.1
III	1.3	0.3	7.8*	0.9	11.3*	0.8
IV	3.2	0.5	5.6	1.3	11.7*	1.4
V	1.7	0.5	8.6*	1.8	13.5*	0.4
VI	3.5	0.6	6.1	1.2	11.8*	0.3
VII	1.6	0.1	2.1	0.3	11.1*	2.4
VIII	2.3	0.1	2.9	0.2	13.3*	0.2
IX	1.5	0.6	1.1	0.4	13.5*	0.9
X	2.2	1.6	2.3	0.3	10.8*	1.5
XI	4.0	1.9	3.3	0.9	11.5*	0.3
XII	3.2	0.8	4.4	0.4	11.3*	2.4

[a] T84 cells were treated with 50 μM of compounds **I**–**XII** or vehicle (DPBS with 0.1 % DMSO) for 1, 24 and 48 hours. Results are normalized to the vehicle group. $n = 3$; Two-Way ANOVA followed by Tukey's test; *$p < 0.05$ compared to vehicle group.

2.5. Measurement of Diarrhea In Vivo in a Model of Rabbit Ileal Loops

In order to investigate the efficacy of compound **VI** in reducing bacterial toxin-induced diarrhea (dependent on the activation of cGMP pathways and / or cAMP) in vivo, as well as the possibility of modulation of the inflammatory process and cell lesions induced by this toxin, we used the technique previously described by Alcantara *et al.*[36] In particular, a midline abdominal incision was made to expose the small bowel of rabbits previously anesthetized. Then 6–8 loops of 3 cm each were ligated and compound **VI** or FPIPP + STa toxin were injected. The in vivo tests were performed with an allowance and according to ethics committee approval.

The data reported in Figure 4 shows that both, compound **VI** and FPIPP are effective in reducing the accumulation of fluid in the intestinal tissue when stimulated by the STa toxin. Statistically, as well as the observations in cell culture, both compounds do not differ from each other.

However, histopathological findings point to a relevant biological difference between the two compounds. According to these results, the treatment with compound **VI** prevents microscopic changes in the intestinal tissue, preserving tissue architecture, highlighting mild mucosal hyperplasia, associated with deposition of mucoid material and rare hematic focus, decreased inflammatory pattern when compared to FPIPP treated tissue, represented by less lymphocytes, plasmocytes and rare eosinophils. The submucosa and lamina propria are preserved, with mild edema and lymphatic dilatation, that can

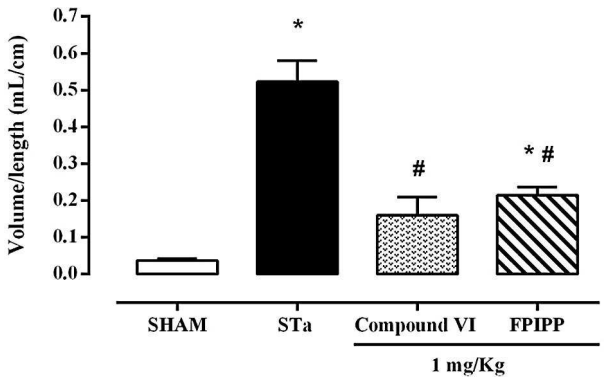


Figure 4. Intraluminal injection of compound **VI** and/or FPIPP (1 mg/loop) in Heat Stable Toxin (STa, 20 μg/mL) treated rabbit ileal loops. *$P < 0.05$ (SHAM vs. STa; SHAM vs. FPIPP); #$P < 0.05$ (STa vs. Compound **VI**; STa vs. FPIPP). One-way ANOVA, complemented by Tukey's test.

be characterized as a hyperplasia of intestinal mucosa associated with lymphoplasmocitary aggregates.

FPIPP compound, however, does not demonstrate such activity in the intestinal tissue. Although it is able to reduce the accumulation of fluids induced by the toxin, there is an intense loss of tissue architecture with features of moderate necrosis and/or accentuated inflammatory aggregates. Also, a significant deposition of serocellular material, with moderate epithelial hyperplasia of the villi, focal area of mucosal dilatation, a multifocal lymphoplasmacytic inflammatory infiltrate, evidencing a moderate arrangement in nests, mixing pronounced suppurative/exudative traits in the middle of the mucosa transition. In the submucosal region, there is moderate tissue edema, with the spacing of the collagen fibers and lymphatic dilatation, that can be characterized as a severe diffuse lymphoplasmocitary enteritis, associated with exudative traits.

2.6. Histopathological Analysis of Intestinal Tissue

The microscopic analysis of stained slides was made in a Nikon binocular microscope (E200). First, slides were placed in microscope stage and illumination adjusted with the aid of the condenser and diaphragm. Then, image was focused with a 10x and 40x objective. Tissue architecture was evaluated throughout the slide and histopathological findings were measured according to the following score:

0) Preserved tissue absent from inflammatory cellularity and necrosis;
1) Mild tissue disorganization with rare inflammatory cells;
2) Moderate tissue disorganization with sketches of necrosis and/or discrete inflammatory infiltrate aggregate;
3) Clear tissue disorganization with mild necrosis traces and/or moderate inflammatory aggregates;
4) Loss of tissue architecture with features of moderate necrosis and/or accentuated inflammatory aggregates;
5) Loss of complete tissue architecture with severe necrosis and/or marked inflammatory aggregates.

The obtained images are reported in Figure 5.

In particular, the microscopic inspection allowed the observation of the tissue modifications due to the exposure to STa toxin alone or in combination with compound **VI** or FPIPP; the experiment results for each case together with the relative anti-inflammatory score are reported as follows:

2.6.1. No Treatment with STa Toxin (Vehicle only)

Histopathological evaluation (Figure 5A) reveals preserved tissue architecture, highlighting mild mucosal edema and deposition of superficial secretory material. In submucosa and muscular layer are observed discrete foci of vascular congestion and edema of the collagen fibers.

Conclusion of analysis: preserved intestine without significant pathological changes, with an inflammatory score of 0 (preserved tissue absent from inflammatory cellularity and necrosis).

2.6.2. Treatment with STa Toxin and Post-Treatment with Vehicle

Histopathological evaluation (Figure 5B) reveals a discrete deposition of serocellular material, with moderate epithelial hyperplasia of the villi, mixing focal area of mucosal dilatation. In the middle of the epithelial layer is observed a multifocal lymphoplasmacytic inflammatory infiltrate, evidencing a moderate arrangement in nests, mixing pronounced suppurative/exudative traits in the middle of the mucosa transition. In the submucosal region, there is moderate tissue edema, with the spacing of the collagen fibers and lymphatic dilatation.

Conclusion of analysis: severe diffuse lymphoplasmocitary enteritis, associated with exudative traits, with an inflammatory score of 4 (loss of tissue architecture with features of moderate necrosis and/or accentuated inflammatory aggregates).

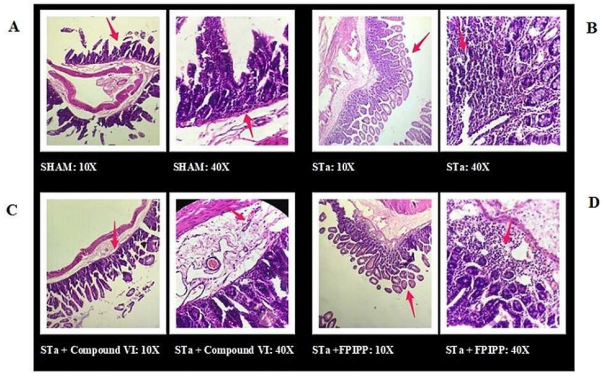


Figure 5. Histopathological analysis of intestinal tissue (ileum) of rabbits treated for 5 hours with: A) vehicle; B) STa toxin; C) Compound **VI** + STa toxin; D) FPIPP + STa toxin.

2.6.3. Pre-Treatment with Compound VI and Post-Treatment with STa Toxin

Histopathological evaluation (Figure 5C) reveals preserved tissue architecture, highlighting mild mucosal hyperplasia, associated with deposition of mucoid material and rare hematic focus. In the middle of the mucosa there is a discrete mixed inflammatory pattern, represented by lymphocytes, plasmocytes and rare eosinophils. The submucosa and lamina propria are preserved, with mild edema and lymphatic dilatation.

Conclusion of analysis: hyperplasia of intestinal mucosa associated with lymphoplasmocitary aggregates, with an inflammatory score of 2 (moderate tissue disorganization with sketches of necrosis and/or discrete inflammatory infiltrate aggregate).

2.6.4. Pre-Treatment with FPIPP and Post-Treatment with STa Toxin

Histopathological evaluation (Figure 5D) reveals a discrete deposition of serocellular material, with moderate epithelial hyperplasia of the villi, mixing focal area of mucosal dilatation. In the middle of the epithelial layer is observed a multifocal lymphoplasmacytic inflammatory infiltrate, evidencing a moderate arrangement in nests, mixing pronounced suppurative/exudative traits in the middle of the mucosa transition. In the submucosal region, there is moderate tissue edema, with the spacing of the collagen fibers and lymphatic dilatation.

Conclusion of analysis: severe diffuse lymphoplasmocitary enteritis, associated with exudative traits, with an inflammatory score of 4 (loss of tissue architecture with features of moderate necrosis and/or accentuated inflammatory aggregates).

2.7. Air Exposure Stability Evaluation for Compound VI

In order to evaluate the chemical stability of compound **VI**, identified as the most active compound towards cGMP accumulation, we decided to perform experiments designed to assess its resistance against air oxidation, both in solid state and in methyl alcohol solution. In particular the procedure was as below reported:

2.7.1. Solid State

Two samples of the same lot of compound **VI** were prepared as follows:

Sample **A**: 10 mg, stored at 4 °C under nitrogen atmosphere.

Sample **B**: 10 mg, room temperature, open vial.

After 4 weeks the samples **A** and **B** were compared by visual inspection, analytical RP-HPLC and ESI-MS. Results, reported in Figures S1–S3 (supplementary materials), suggest no oxidation of the compound.

2.7.2. Methyl Alcohol Solution

Two samples of the same lot of compound **VI** were prepared as follows:

Sample **C**: 1 mg, diluted in 1 mL of MeOH, stored at 4 °C under nitrogen atmosphere.

Sample **D**: 1 mg, diluted in 1 mL of MeOH, room temperature, open vial.

After 4 weeks the samples were compared by visual comparison (Figure S4), analytical RP-HPLC analyses and by LC-HRMS.

Analytical HPLC results evidenced that prolonged storage of a methanolic solution of the compound **VI**, at room temperature and under air exposure, allowed the formation of a small amount of two by-products, named **"a"** and "**b**" (Figure S5).

Sample **D** was therefore further investigated by LC-HRMS in order to characterize these compounds.

By-product "**a**" revealed a molecular weight of 538.1189 corresponding to a molecule having a general formula of $C_{25}H_{17}O_4N_3F_6$; we propose that this compound corresponds to the N-hydroxy derivative of compound **VI**. By-product "**b**", characterized by a molecular weight of 520.1081, has been identified as a pyridine derivative, obtained by oxidation of compound **VI**. The chemical structure of compounds "**a**" and "**b**" are showed in Figure S6.

3. Conclusions

Research results reported in this work allowed the identification of pyridopyrimidines acting as a promising class of antidiarrheal agents. The molecules were prepared by an optimized synthetic methodology that was found as the best strategy for the obtaining all compounds in high yields and purity. The most notable derivatives were compounds **VI** and **IV**, with a partial selectivity towards cGMP and cAMP, respectively. In vitro assays demonstrated that none of the synthesized compounds affects vascular tonus of pre-contracted aortic rings when compared to amlodipine. Moreover, the metabolic activity and viability in T84 cells were not affected by compounds **I**–**XII** as MTT and LDH assays evidenced.

Compound **VI** was then selected for a specific in vivo insight focused to the evaluation of its anti-diarrheal activity in rabbits; the experiments performed on rabbit ileum ligations evidenced a potent effect of compound **VI** and FPIPP. This prompted us to perform a microscopic histopathological analysis of intestinal tissues aimed to the evaluation of tissue architecture. The images allowed to reveal that only compound **VI** preserves intestine, without significant pathological changes and with a decreased inflammatory pattern in comparison to FPIPP. Finally, bearing in mind that the oxidative aromatization of 1,4-DHPs to the corresponding pyridines has been extensively studied and that a plethora of oxidants including air have been applied in the aromatization reactions, compound **VI** was further investigated for its chemical stability and revealed to be suitable for a prolonged storage in solid state. This derivative therefore candidates as "lead compound" of this class of pyridopyrimi-

dines, an interesting scaffold for the generation of molecules for possible use as efficient anti-diarrheal drugs.

Experimental Section

Chemistry

5-(Trifluoromethyl)thiophene-3-carbaldehyde was purchased from Aurora Building Blocks, 1-ethyl-3-methylurea was purchased from AKos Building Blocks and all the other commercial products have been purchased from Sigma Aldrich. All the reactions have been followed by TLC, carried out on Merck silica gel 60 F254 (E. Merck, AG, Darmstadt, Germany) plates with fluorescent indicator and the plates have been visualized with UV light (254 nm). Microwave reactions were performed using a microwave oven (ETHOS 1600, Milestone) especially designed for organic synthesis. Microwave reactions were performed in sealed tubes and were performed by microwave program which was composed by appropriate ramping and holding steps. Identification of the optimum profile power/time and temperature for the synthesis was preliminarily assessed. The temperature of the stirred reaction mixture was monitored directly by a microwave-transparent fluoroptic probe inserted into the reaction mixture; irradiation time and power were monitored with the "easyWAVE" software package. No further yield increasing was evidenced when the irradiation power or time were increased due to decomposition of the starting materials. Preparative chromatographic purifications have been performed using silica gel column (Kieselgel 60). Solutions have been concentrated with a Büchi rotary evaporator at low pressure. Melting points, determined using a Büchi Melting Point B-540 instrument, are uncorrected and represent values obtained on recrystallized or chromatographically purified material. Molecular weights of intermediates have been assessed by electrospray ionization mass spectrometry (ESI/MS) performed on an API 2000 Applied Biosystems mass spectrometer. Analytical RP-HPLC analyses were performed using a Phenomenex Kinetex C18 column (2.5 μm, 4.6×300 mm). The column was connected to a Rheodyne model 7725 injector, a Shimadzu-10 ADsp HPLC system, a Shimadzu SPD-20 A/SPD-20 AV UV-VIS detector set to 254 nm. The analytical determinations employed solvent A: 100% acetonitrile in 0.1% TFA, and B: 100% H_2O in 0.1% TFA. The operational conditions involved a linear gradient of 0–100% acetonitrile with 0.1% TFA, which developed in 30 minutes at a flow rate of 1 mL/min. LC-HRMS was carried out on a Dionex Ultimate 3000 quaternary system coupled to a hybrid linear ion trap LTQ Orbitrap XLTM Fourier Transform MS (FTMS) equipped with an ESI ION MAXTM source (Thermo-Fisher, San Josè, CA, USA). The same instrument was used for HRMS (infusions) of final compounds. Chromatographic separation was accomplished by using a Kinetex C18 column (100×2.1 mm, 1.7 μm) (Phenomenex, Le Pecq, France) maintained at room temperature and eluted at 0.2 mL min^{-1} with water (eluent A) and 95% acetonitrile/water (eluent B), both containing 30 mM acetic acid. The ^1H and ^{13}C-NMR spectra have been recorded on a Varian Mercury Plus 400 MHz instrument. All spectra have been recorded in DMSO-d_6. The following abbreviations are used to describe peak patterns when appropriate: s (singlet), bs (broad singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), m (multiplet).

Analytical purification of each product was obtained by silica gel column chromatography and by crystallization from the appropriate solvent. Analyses indicated by the symbols of the elements were whitin ±0.4 of the theoretical values. ^1H NMR, ^{13}C NMR and MS data for all final compounds were consistent with the proposed structures. Representative HRMS, ^1H and ^{13}C-NMR spectra are available in Supplementary Materials (Figures 7 S, 8 S and 9 S, respectively).

6-Amino-1,3-Dimethyl-2-Thioxo-2,3-Dihydropyrimidin-4(1H))-One (3 b)

To a stirred solution of 1,3-dimethylthiourea (1.83 g, 17.6 mmol) in acetic anhydride (30 mL) was added cyanoacetic acid (1.5 g, 17.6 mmol), and the resulting mixture was transferred into a sealed vessel and heated by microwave irradiation at 70 °C (200 W power) for 2 hours. The reaction mixture was concentrated and the resulting oily residue was diluted with H_2O (40 mL) and treated with 5 N NaOH (15 mL). The precipitate thus formed was collected by filtration, washed with cold water and purified by recrystallization from MeOH/H_2O to give 2.65 g of 6-amino-1,3-dimethyl-2-thioxo-2,3-dihydropyrimidin-4(1H)-one.

Yield: 2.86 g, 95%. m.p. 289 °C. ^1H-NMR (400 MHz, DMSO-d_6): δ 7.01 (s, 2H, NH2), 5.01 (s, 1H), 3.74 (s, 3H), 3.50 (s, 3H). ESI-MS (MW 171.05): 172.1 ($M+H^+$). Anal. calcd for $C_6H_9N_3OS$: C 42.09, H 5.30, O 9.34, found: C 42.49, H 5.00, O 9.44.

Synthesis of 6-Amino-1-Ethyl-3-Methylpyrimidine-2,4(1H, 3H)-Dione (3 c)

To a stirred solution of 1-ethyl-3-methylurea (1.36 g, 13.3 mmol) in acetic anhydride (30 mL) was added cyanoacetic acid (1.13 g, 13.3 mmol), and the resulting mixture was transferred into a sealed vessel and heated by microwave irradiation at 70 °C (200 W power) for 2 hours. The reaction mixture was concentrated and the resulting oily residue was diluted with H_2O (40 mL) and treated with 5 N NaOH (15 mL). The precipitate thus formed was collected by filtration, washed with cold water and purified by recrystallization from MeOH/H_2O to give 1.91 g of 6-amino-1-ethyl-3-methylpyrimidine-2,4(1H,3H)-dione.

Yield: 2.11 g, 94%. m.p. 232 °C. ^1H-NMR (400 MHz, DMSO-d_6): δ 6.76 (s, 2H, NH2), 4.64 (s, 1H), 3.80 (q, 2H), 3.29 (s, 3H), 1.08 (t, 3H). ESI-MS (MW 169.09): 170.1 $[M+H]^+$. Anal. calcd for $C_7H_{11}N_3O_2$: C 49.70, H 6.55, O 18.91, found: C 49.30, H 6.15, O 18.71.

5-(4-Trifluoromethylthiophen)-1,3-Dimethyl-5,11-Dihydro-1-H-Indeno-[2′,1′:5,6]Pyrido[2,3-d]Pyrimidine-2,4,6-Trione (I)

To acetic acid (15 mL) in a round bottom flask, 1,3-indandione (**1**, 0.56 g, 3.87 mmol), 5-(trifluoromethyl)thiophene-3-carbaldehyde (**2 c**, 0.697 g, 3.87 mmol) and 6-amino-1,3-dimethyluracil (**3 a**, 0.5 g, 3.22 mmol) were added. T The mixture was then transferred into a sealed vessel and heated by microwave irradiation at 120 °C (400 W power) for 40 minutes (2 min ramping step; 38 min holding step). It was then cooled to 0 °C. The resulting precipitate was filtered and rinsed with cold water to obtain the crude product. Purification on silica gel column (CH_2Cl_2/CH_3OH, 9,5/0,5, v/v) afforded the desired compound **I** as an orange powder. Yield: 1.28 g, 91%. m.p. 265 °C. ^1H-NMR (400 MHz, DMSO-d_6): δ 9.88 (s, 1H, NH), 7.85 (d, 1H), 7.59 (d, 1H), 7.56 (s, 1H), 7.46 (t, 1H), 7.32 (t, 1H), 5.73 (s, 1H), 4.89 (s, 1H), 3.56 (s, 3H), 3.13 (s, 3H). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 190.9, 161.5, 155.5, 154.5, 151.1, 146.8, 133.6, 132.6, 130.8, 130.6, 127.0, 124.0, 122.1, 121.5, 121.4, 121.3, 108.9, 91.2, 31.3, 30.0, 29.0. HRMS 445.93459. Anal. calcd for $C_{21}H_{14}F_3N_3O_3S$: C 56.63, H 3.17, O 10.78, found: C 56.43, H 3.07, O 10.98.

5-(4-Trifluoromethylthiophen)-1,3-Dimethyl-5,11-Dihydro-1-H-Indeno-[2′,1′:5,6]Pyrido[2,3-d] Pyrimidine-2-Thioxo-4,6-Dione (II)

The compound was obtained with the same procedure described for compound **I**, by reaction of 1,3-indandione (**1**, 170 mg, 1.16 mmol), 5-(trifluoromethyl)thiophene-3-carbaldehyde (**2c**, 209 mg, 1.16 mmol) and 6-amino-1,3-dimethyl-2-thioxo-2,3-dihydropyrimidin-4(1H)-one (**3b**, 166 mg, 0.97 mmol). Yield: 0.335 g, 75%. m.p. 178 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.09 (s, 1H, NH), 7.87 (d, 1H), 7.66 (d, 1H), 7.60 (t, 1H), 7.49 (s, 1H), 7.33 (t, 1H), 5.95 (s, 1H), 4.96 (s, 1H), 4.07 (s, 3H), 3.58 (s, 3H). ^{13}C-NMR (100 MHz, CD_3OD): δ 193.2, 178.3, 161.5, 155.4, 152.2, 146.8, 146.8, 133.5, 131.5, 130.7, 130.6, 126.9, 126.9, 122.4, 121.1, 121.0, 107.8, 97.2, 39.0, 36.2, 31.3. HRMS 461.95812. Anal. calcd for $C_{21}H_{14}F_3N_3O_2S_2$: C 54.66, H 3.06, O 6.93, found: C 54.36, H 3.36, O 6.63.

5-(3-Fluoro-5-trifluoromethylphenyl)-1,3-dimethyl-5,11-dihydro-1H-indeno-[2′,1′:5,6] pyrido[2,3-d] pyrimidine-2-thioxo-4,6-dione (III)

The compound was obtained with the same procedure described for compound **I**, by reaction of 1,3-indandione (**1**, 1.11 g, 7.61 mmol), 3-fluoro-5-trifluoromethylbenzaldehyde (**2b**, 1.46 g, 7.61 mmol) and 6-amino-1,3-dimethyl-2-thioxo-2,3-dihydropyrimidin-4(1H)-one (**3b**, 1.85 g, 6.34 mmol). Yield: 2.01 g, 67%. m.p. 313 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.09 (s, 1H, NH), 8.03 (d, 1H), 7,66 (s, 1H), 7,54 (s, 1H), 7,51 (d, 1H), 7.41 (t, 1H), 7,35 (t, 1H), 7,28 (s, 1H), 4,96 (s, 1H), 4.08 (s, 3H), 3.52 (s, 3H). ^{13}C-NMR (100 MHz, CD_3OD): δ 193.2, 178.0, 167.5, 158.4, 156.8, 154.9, 153.0, 150.2, 143.9, 138.8, 136.6, 134.7, 133.9, 127.1, 124.3, 123.1, 122.6, 122.2, 120.4, 109.6, 92.6, 38.1, 36.0. HRMS 474.10597. Anal. calcd for $C_{23}H_{15}F_4N_3O_2S$: C 58.35, H 3.19, O 6.76, found: C 58.55, H 3.29, O 6.86.

5-(3-Fluoro-5-trifluoromethylphenyl)-1-ethyl-3-methyl-5,11-dihydro-1H-indeno-[2′,1′:5,6] Pyrido[2,3-d] pyrimidine-2,4,6-trione (IV)

The compound was obtained with the same procedure described for compound **I**, by reaction of 1,3-indandione (**1**, 970 mg, 6.64 mmol), 3-fluoro-5-trifluoromethylbenzaldehyde (**2b**, 1.27 g, 6.64 mmol) and 6-amino-1-ethyl-3-methylpyrimidine-2,4(1H,3H)-dione (**3c**, 936 mg, 5.53 mmol). Yield: 1.64 g, 64%. m.p. 177 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.81 (s, 1H, NH), 7.94 (d, 1H), 7.49 (m, 3H), 7.37 (d, 1H), 7.33 (t, 1H), 7.27 (t, 1H), 4.91 (s, 1H), 4.26 (m, 2H), 3.10 (s, 3H), 1.24 (t, 3H). ^{13}C-NMR (100 MHz, CD_3OD): δ 193.2, 165.1, 163.4, 162.7, 155,7, 152.0, 150.6, 150.5, 146.4, 137.5, 133.9, 133.5, 131.5, 126.1, 122.3, 121.7, 121.1, 119.7, 119.5, 111.5, 93.2, 36.1, 28.4, 13.8. HRMS 471.12632. Anal. calcd for $C_{24}H_{17}F_4N_3O_3$: C 61.15, H 3.63, O 10.18, found: C 61.25, H 3.43, O 10.38.

5-(3,5-Bistrifluoromethylphenyl)-1,3-dimethyl-5,11-dihydro-1-H-indeno-[2′,1′:5,6]pyrido[2,3-d] pyrimidine-2-thioxo-4,6-dione (V)

The compound was obtained with the same procedure described for compound **I**, by reaction of 1,3-indandione (**1**, 503 mg, 3.44 mmol), 3,5-bistrifluoromethylbenzaldehyde (**2a**, 833 mg, 3.44 mmol) and 6-amino-1,3-dimethyl-2-thioxo-2,3-dihydropyrimidin-4(1H)-one (**3b**, 490 mg, 2.87 mmol). Yield: 1.16 g, 72%. m.p. 314 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.14 (s, 1H, NH), 8.08 (d, 1H), 7.97 (s, 2H), 7.87 (s, 1H), 7.53 (t, 1H), 7.37 (t, 1H), 7.25 (d, 1H), 5.06 (s, 1H), 4.08 (s, 3H), 3.51 (s, 3H). ^{13}C-NMR (100 MHz, DMSO-d_6): 187.4, 177.0, 166.9, 157.6, 153.9, 146.8, 139.5, 136.7, 135.6, 135.3, 132.9, 128.9, 128.6, 127,9, 123.3, 121.6, 108.6, 93.7, 37.3, 35.0, 27.7. HRMS 524.03679. Anal. calcd for $C_{24}H_{15}F_6N_3O_2S$: C 55.07, H 2.89, O 6.11, found: C 55.37, H 2.99, O 6.41.

5-(3,5-Bistrifluoromethylphenyl)-1-ethyl3-methyl-5,11-dihydro-1H-indeno-[2′,1′:5,6] Pyrido[2,3-d] pyrimidine-2,4,6-trione (VI)

The compound was obtained with the same procedure described for compound **I**, by reaction of 1,3-indandione (**1**, 708 mg, 4.85 mmol), 3,5-bistrifluoromethylbenzaldehyde (**2a**, 1.17 g, 4.85 mmol) and 6-amino-1-ethyl-3-methylpyrimidine-2,4(1H,3H)-dione (**3c**, 683 mg, 4.04 mmol). Yield: 2.01 g, 85%. m.p. 166 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.87 (s, 1H, NH), 7.94 (d, 1H), 7.91 (s, 2H), 7.88 (s, 1H), 7.50 (t, 1H), 7.37 (t, 1H), 7.30 (d, 1H), 5.03 (s, 1H), 4.32 (m, 2H), 3.09 (s, 3H), 1.24 (t, 3H). ^{13}C-NMR (100 MHz, DMSO-d_6): 191.3, 168.1, 161.4, 154.8, 150.7, 148.6, 145.3, 136.2, 132.7, 132.5, 131.0, 130.5, 128.9, 125.2, 121.5, 121.4, 120.8, 109.3, 91.2, 35.5, 28.1, 14.0. HRMS 522.12598. Anal. calcd for $C_{25}H_{17}F_6N_3O_3$: C 57.59, H 3.29, O 9.21, found: C 57.69, H 3.39, O 9.31.

5-(Benzo[d][1,3] dioxol-4-yl)-1,3-dimethyl-5,11-dihydro-1H-indeno-[2′,1′:5,6] pyrido[2,3-d]pyrimidine-2,4,6-trione (VII)

The compound was obtained with the same procedure described for compound **I**, by reaction of 1,3-indandione (**1**, 1.61 g, 11.06 mmol), 2,3-(methylenedioxy)benzaldehyde (**2g**, 1.66 g, 11.06 mmol) and 6-amino-1,3-dimethyluracil (**3a**, 1.43 g, 9.22 mmol). Yield: 3.44 g, 90%. m.p. 300 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.88 (s, 1H, NH), 7.83 (d, 1H), 7.45 (t, 1H), 7.32 (t, 1H), 7.24 (d, 1H), 6.73 (t, 1H), 6.66 (d, 2H), 5.90 (d, 2H), 4.81 (s, 1H), 3.56 (s, 3H), 3.08 (s, 3H). ^{13}C-NMR (100 MHz, DMSO-d_6): 188.1, 168.2, 159.4, 155.9, 150.7, 146.8, 145.7, 144.2, 140.7, 136.4, 136.0, 133.6, 124.7, 124.0, 122.3, 120.9, 120.5, 116.4, 108.5, 107.6, 101.1, 30.8, 28.6. HRMS 416.08251. Anal. calcd for $C_{23}H_{17}N_3O_5$: C 66.50, H 4.12, O 19.26, found: C 66.90, H 4.52, O 19.66.

5-(Benzo[d][1,3] dioxol-4-yl)-1,3-dimethyl-5,11-dihydro-1H-indeno-[2′,1′:5,6] pyrido[2,3-d]pyrimidine-2-thioxo-4,6-dione (VIII)

The compound was obtained with the same procedure described for compound **I**, by reaction of 1,3-indandione (**1**, 535 mg, 3.66 mmol), 2,3-(methylenedioxy)benzaldehyde (**2g**, 549 mg, 3.66 mmol) and 6-amino-1,3-dimethyl-2-thioxo-2,3-dihydropyrimidin-4(1H)-one (**3b**, 521 mg, 3.05 mmol). Yield: 1.02 g, 78%. m.p. 310 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.11 (s, 1H, NH), 7.83 (d, 1H), 7.47 (t, 1H), 7.34 (t, 1H), 7.27 (d, 1H), 6.76 (t, 1H), 6.68 (d, 2H), 5.91 (d, 2H), 4.88 (s, 1H), 4.08 (s, 3H), 3.52 (s, 3H). ^{13}C-NMR (100 MHz, DMSO-d_6): 193.5, 179.3, 161.5, 156.8, 149.8, 148.8, 148.18, 147.2, 143.1, 139.3, 135.2, 133.3, 129.2, 125.3, 124.0, 123.8, 112.0, 111.4, 110.0 103. 6, 98.5, 38.4, 33.0. HRMS 432.04328. Anal. calcd for $C_{23}H_{17}N_3O_4S$: C 64.03, H 3.97, O 14.83, found: C 64.23, H 3.67, O 15.03.

5-(Benzo[d][1,3] dioxol-5-yl)-1,3-dimethyl-5,11-dihydro-1H-indeno-[2′,1′:5,6] pyrido[2,3-d]pyrimidine-2,4,6-trione (IX)

The compound was obtained with the same procedure described for compound **I**, by reaction of 1,3-indandione (**1**, 203 mg, 1.39 mmol), 3,4-(methylenedioxy)benzaldehyde (**2f**, 209 mg, 1.39 mmol) and 6-amino-1,3-dimethyluracil (**3a**, 180 mg,

1.16 mmol). Yield: 0,352 g; 73%. m.p. 338 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.79 (s, 1H, NH), 7.98 (d, 1H), 7.86 (t, 1H), 7.77 (t, 1H), 7.62 (d, 1H), 6.92 (d, 1H), 6.81 (s, 1H), 6.72 (d, 1H), 5.89 (s, 2H), 4.69 (s, 1H), 3.57 (s, 3H), 3.10 (s, 3H). ^{13}C-NMR (100 MHz, DMSO-d_6): 189.2, 168.5, 159.9, 156.5, 151.5, 146.8, 145.7, 145.2, 140.9, 136.8, 135.9, 133.3, 124.6, 124.3, 122.9, 121.2, 120.3, 117.0, 109.1, 108.0, 101.1, 31.1, 27.9. HRMS 416.06943. Anal. calcd for $C_{23}H_{17}N_3O_5$: C 66.50, H 4.12, O 19.26, found: C 66.70, H 4.02, O 19.16.

5-(2,1,3-Benzothiadiazol-4-yl)-1,3-dimethyl-5,11-dihydro-1H-indeno-[2′,1′:5,6]pyrido[2,3-d]pyrimidine-2,4,6-trione (X)

The compound was obtained with the same procedure described for compound **I**, by reaction of 1,3-indandione (**1**, 420 mg, 2.87 mmol), 2,1,3-benzothiadiazol-4-carboxaldehyde (**2e**, 471 mg, 2.87 mmol) and 6-amino-1,3-dimethyluracil (**3a**, 359 mg, 2.39 mmol). Yield: 0.82 g, 80%. m.p. 331 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.08 (s, 1H, NH), 7.88 (d, 1H), 7.62 (d, 3H), 7.50 (t, 1H), 7.32 (t, 1H), 7.23 (d, 1H), 5.46 (s, 1H), 3.63 (s, 3H), 3.01 (s, 3H). ^{13}C-NMR (100 MHz, DMSO-d_6): 191.3, 168.1, 161.3, 155.2, 155.0, 153.7, 151.0, 146.00, 140.7, 138.1, 132.7, 132.4, 130.6, 128.9, 121.2, 121.1, 121.0, 119.9, 109.7, 91.8, 32.9, 27.5. HRMS 430.15267. Anal. calcd for $C_{22}H_{15}N_5O_3S$: C 61.53, H 3.52, O 11.18, found: C 61.63, H 3.42, O 11.48.

5-(2,1,3-Benzothiadiazol-5-yl)-1,3-dimethyl-5,11-dihydro-1H-indeno-[2′,1′:5,6]pyrido[2,3-d]pyrimidine-2,4,6-trione (XI)

The compound was obtained with the same procedure described for compound **I**, by reaction of 1,3-indandione (**1**, 468 mg, 3.20 mmol), 2,1,3-benzothiadiazol-5-carboxaldehyde (**2d**, 525 mg, 3.2 mmol) and 6-amino-1,3-dimethyluracil (**3a**, 415 mg, 2.67 mmol). Yield: 1.08 g, 94%. m.p. 358 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.92 (s, 1H, NH), 7.90 (m, 3H), 7.77 (d, 1H), 7.47 (t, 1H), 7.33 (t, 1H), 7.26 (d, 1H), 4.99 (s, 1H), 3.61 (s, 3H), 3.08 (s, 3H).^{13}C-NMR (100 MHz, DMSO-d_6): 194.1, 163.8, 157.7, 157.2, 156.6, 153.7, 150.0, 148.4, 139.1, 138.6, 136.3, 135.0, 134.8, 134.2, 133.2, 126.5, 123.5, 121.7, 112.4, 93.7, 34.1, 30.7. HRMS 430.11667. Anal. calcd for $C_{22}H_{15}N_5O_3S$: C 61.53, H 3.52, O 11.18, found: C 61.73, H 3.22, O 11.28.

5-(2,1,3-Benzothiadiazol-5-yl)-1,3-dimethyl-5,11-dihydro-1H-indeno-[2′,1′:5,6]pyrido[2,3-d]pyrimidine-2-thioxo-4,6-dione (XII)

The compound was obtained with the same procedure described for compound **I**, by reaction of 1,3-indandione (**1**, 456 mg, 3.12 mmol), 2,1,3-benzothiadiazol-5-carboxaldehyde (**2d**, 512 mg, 3.12 mmol) and 6-amino-1,3-dimethyl-2-thioxo-2,3-dihydropyrimidin-4(1H)-one (**3b**, 445 mg, 2.60 mmol). Yield: 1.11 g, 96%. m.p. 362° C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.15 (s, 1H, NH), 7.96 (m, 3H), 7.80 (d, 1H), 7.48 (t, 1H), 7.34 (t, 1H), 7.27 (d, 1H), 5.05 (s, 1H), 4.11 (s, 3H), 3.52 (s, 3H). ^{13}C-NMR (100 MHz, DMSO-d_6): 190.9, 176.4, 159.5, 155.0, 154.3, 153.7, 147.3, 146.6, 136.8, 136.6, 133.7, 132.6, 132.1, 131.0, 130.9, 121.4, 121.3, 120.9, 119.5, 96.0, 35.6, 21.3. HRMS 446.07465. Anal. calcd for $C_{22}H_{15}N_5O_2S_2$: C 59.31, H 3.39, O 7.18, found: C 59.21, H 3.29, O 7.28.

Biological Experiments

Cell Culture

Human colorectal carcinoma cell, or T84 cell, was from the American Type Culture Collection (CCL248) and were grown in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (Sigma-Aldrich, #D8900) supplemented with 10% FBS (Gibco, #12657029) and penicillin-streptomycin (Gibco, #15140122) in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. Jurkat E6-1 cells were purchased from APABCAM (BCRJ, #0125) and were grown in RPMI 1640 medium (Gibco, #61870-036) supplemented with 10% FBS (Gibco, #12657029) and penicillin-streptomycin (Gibco, #15140122) in a humidified atmosphere of 95% air/5% CO_2 at 37 °C.

cGMP Accumulation in Cells

T84 cells were grown to confluence in 12-well plates (Santa Cruz Biotechnology, #sc-204444) and washed three times with DPBS (pH 7.4). Cells were treated with vehicle (DPBS with DMSO [Sigma-Aldrich, #D2650], 0.1%, 0.5 mL per well) or compounds (50 μM) in DPBS for 10 minutes. Then, cGMP accumulation was stimulated with enterotoxin of *E. Coli* (STa, 1 μM [Bachem, #404429]) for 10 minutes. Medium was aspirated and cGMP was extracted with 0.1 M HCl (0.3 mL per well) and measured by ELISA (acetylation protocol; Cayman Chemical, #581021).

AMP Accumulation in Cells

Jurkat cells were grown to 5 million cells/mL in 75 cm^2 culture bottle (Santa Cruz Biotechnology, #sc-200263). The cells were centrifuged at 3000×g for five minutes and washed three times with DPBS (pH 7.4). Jurkat cells (0.1 mL of cell suspension) were treated with vehicle (DPBS with DMSO 0.1% [Sigma-Aldrich, D2650], 0.9 mL per vial) or compounds (50 μM) in DPBS for 10 minutes. Then, cAMP accumulation was stimulated with Forskolin 100 μM (Sigma-Aldrich, #F6886) for 10 minutes. cAMP was extracted with 0.2 M HCl (0.1 mL per vial) and gentle shaking on an orbital shaker for 20 minutes at room temperature. After, cells were centrifuged at 3000×g for five minutes and the supernatant frozen at −20 °C. cAMP was measured by ELISA (acetylation protocol; Cayman Chemical, #581001).

Preparation of Rabbit Isolated Aorta

Male rabbits (3 Kg) were anesthetized with ketamine (50 mg/kg), isoflurane (2%) and exsanguinated. Thoracic aorta was isolated and placed in cold Krebs solution (pH 7.4). The composition of the Krebs solution was: NaCl (139.9 mM), KCl (2.7 mM), $CaCl_2$ (1.8 mM); $MgSO_4$ (0.6 mM); $NaHCO_3$ (25 mM), KH_2PO_4 (0.5 mM) and Glucose (11.5 mM). The tissue was dissected and cut in aortic rings (3 mm). Each ring was stretched to 20 mN during 45 minutes for stabilization. Following the stabilization period, the absence of endothelium was evaluated in lack of acetylcholine (1 μM) induce relaxation in ring pre contracted with phenylephrine (1 μM). After washing, the rings were pre contracted with a hyperpolarization solution of KCl (60 mM) and the effects of compounds were evaluated.

MTT Assay (Cell Metabolic Activity)

Using 96-well plates, T84 cells were grown for 48 h according to the supplier guidelines in the presence of 10% FBS in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. Medium was aspirated and replaced by medium protein free (100 μl/well). Compounds (50 μM) and vehicle (Dulbecco's PBS with DMSO 0.1% [Sigma-Aldrich, #D2650]) were incubated for 1 hour. After incubation, medium was aspirated and replaced by medium protein free (100 μl/well) and 10 μl MTT (Sigma-Aldrich, #M2128) solution (final concentration at 0.45 mg/mL). Plates were incubated for 2 to 4 hours at 37 °C and medium aspirated and replaced by 100 μl of solubilization solution

(acidified isopropanol), mixed by 15 minutes and absorbance read at 570 nm.

LDH Cytotoxicity Assay

Cytotoxicity was measured using CaymanChem LHD Assay Kit (#601170). In summary, T84 cells were grown in a 96-well plate at the previously determined optimal density in 200 µl of culture medium. Triton X-100 (10%; 20 µl) was added to wells containing cells to get maximum release; Assay buffer (20 µl) was added to get spontaneous release and LDH (20 µl) was added as positive control. The compounds (50 µM) or its vehicle were added (20 µl) and plate incubated in a CO_2 incubator at 37 °C. Following, plates were centrifuged at 400×g for five minutes and 100 µl of cell supernatant was transferred to a new 96-well assay plate where 100 µl of reaction solution was added to each well. Plates were incubated with gentle shaking on an orbital shaker for 30 minutes at room temperature and the absorbance read at 490 nm.

Rabbit Ileal Loops

Six New Zealand white rabbits weighing 2 kg were fasted overnight. The rabbits were anesthetized with ketamine (60–80 mg/kg) and xylazine (5–10 mg/kg), administered intramuscularly, and a midline abdominal incision was made to expose the small bowel. After that, 6–8 loops of 3 cm each were ligated using double ties, and a 1-cm interval was left between loops.

Each of the control loops was injected intraluminal with a 1-mL solution of PBS with either *E. coli* heat-stable toxin (STa; Sigma-Aldrich). Immediately before the enterotoxin was administered, loops were treated with Compound **VI** or FPIPP were diluted in PBS to concentrations of 1 mg/Kg. The ileal loops were replaced intraperitoneally, and the abdominal incision was sutured closed. The rabbits were maintained under anesthesia until they were euthanized 4 h later.

Measurement of Intestinal Secretion

After 4 h of incubation, the ligated ileal loops were removed. The length of each ligated ileal segment was measured. Intraluminal fluid was extracted from each ileal loop and quantified. The volume: length ratio (V:L) was calculated in milliliters per centimeter per loop. The gross description (clear, serous, purulent, or bloody) of the collected fluid was also noted.

Tissue Fixation and Histologic Coloration

In order to fix the samples, dissected ileum tissues have been placed in labeled 20 mL snap-cap glass vials. Vials have been filled with freshly prepared 4% PFA fixative, 4 °C. Fixation was allowed to proceed at 4 °C for 24 h.

After fixation was completed, fixative solution was replaced with 50% ethanol. Ethanol was substituted with fresh one. Tissues were incubated for 20 min and then 50% ethanol was replaced again. A total of three changes of ethanol, each followed by a 20 min incubation at room temperature, was done in this way. Dehydration was continued by incubating the samples three times in 70% ethanol, 20 min each time (room temperature). Successively, samples were incubated in 95% ethanol for 20 min at room temperature for three times. Finally, incubation in 100% ethanol for 20 min at room temperature was performed three times. Ethanol was replaced by xylene and incubation for 10 min was repeated for three times (each with fresh xylene).

An equal amount of molten wax was added using a hot glass pipet. Samples were mixed and leaved at room temperature overnight. The wax/xylenes mixture hardened. Samples were transferred to 60 °C oven in order to melt the wax/xylenes mixture. Vials were transferred to a heating block and wax/xylenes mixture was replaced. Vials were then incubated for 1 h at 60 °C and moved from oven to heating block again.

Embedding molds were prepared according to manufacturer's instructions and filled with molten paraffin wax using a hot glass pipet. Sample were immediately transferred to the wax-filled mold using a hot cut-off Pasteur pipet. An embedding ring was placed on the mold and fill with paraffin wax. Cast blocks were leaved at room temperature to harden completely and stored in a dry place at room temperature.

A wax block containing samples was cut into a trapezoidal shape using a razor blade. The extra wax was shaved off and the trapezoid block was attached to the holding clamp of a microtome in order to be sectioned, cutting at 8-µm sections. A drop of 0.2×gelatin was putted subbing solution on a gelatin-subbed glass slid and the ribbon of sections were then transferred onto the drop on the subbed slide using fine brushes. The slides were transferred onto a slide warmer or heating plate set between 45° and 50 °C. When stretching was complete slides were removed from the warmer and the remaining subbing solution was carefully removed using a Pasteur pipet. Slides were dried at room temperature and incubated for 2 days at 42 °C to firmly attach sections to subbed slides. Sections have been stored in a slide box with desiccant at −20 °C.

The cryosections slides have successively been immersed in H_2O for 30 seconds with agitation by hand. The slides have been dipped into a Coplin jar containing Mayer's hematoxylin and agitated for 30 seconds. Then the slide have been stained with 1% eosin Y solution for 10–30 seconds with agitation. The sections have been dehydrated with two changes of 95% alcohol and two changes of 100% alcohol for 30 seconds each. The alcohol was extracted with two changes of xylene and finally one or two drops of mounting medium were added, before to cover with a coverslip.

Statistical Analysis

The Kolmogorov-Smirnov test was applied to determine the normal distribution of the data ($p > 0.05$). Data were analyzed using one-way ANOVA complemented by the Tukey post hoc test. The results are expressed as mean + SEM. Statistically significant differences were set at $p < 0.05$. All experiments were repeated at least three times in duplicate.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: pyridopyrimidines · diarrhea · cyclic nucleotides · drug discovery · structure-activity relationships

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