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Effects of agmatine accumulation in human colon carcinoma cells on polyamine metabolism, DNA synthesis and the cell cycle

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Abstract

Putrescine, spermidine and spermine are low molecular polycations that play important roles in cell growth and cell cycle progression of normal and malignant cells. Agmatine (1-amino-4-guanidobutane), another polyamine formed through arginine decarboxylation, has been reported to act as an antiproliferative agent in several non-intestinal mammalian cell models. Using the human colon adenocarcinoma HT-29 $Glc^{-/+}$ cell line, we demonstrate that agmatine, which markedly accumulated inside the cells without being metabolised, exerted a strong cytostatic effect with an IC50 close to 2 mM. Agmatine decreased the rate of L-ornithine decarboxylation and induced a 70% down-regulation of ornithine decarboxylase (ODC) expression. Agmatine caused a marked decrease in putrescine and spermidine cell contents, an increase in the N_1-acetylspermidine level without altering the spermine pool. We show that agmatine induced the accumulation of cells in the S and G_2/M phases, reduced the rate of DNA synthesis and decreased cyclin A and B_1 expression. We conclude that the anti-metabolic action of agmatine on HT-29 cells is mediated by a reduction in polyamine biosynthesis and induction in polyamine degradation. The decrease in intracellular polyamine contents, the reduced rate of DNA synthesis and the cell accumulation in the S phase are discussed from a causal perspective.

Keywords: Agmatine; Polyamine; Cell cycle; Cyclin A; Cyclin B_1; Metabolism

1. Introduction

Colonic epithelial cells are exposed to numerous dietary compounds and to related metabolites from both luminal and basolateral poles which may influence cell physiology [1,2]. Among them are polyamines, low molecular biogenic polycationic amines such as "classical" polyamines and agmatine, found in food from vegetal and animal origins and in fermented food products [3,4]. Polyamines are also produced by luminal colonic bacteria [5].

The "classical" polyamines (putrescine, spermidine, and spermine) are ubiquitous components of mammalian tissues and play essential roles in cell growth and differentiation [6,7]. Putrescine, the essential precursor of the higher polyamines, spermidine and spermine, is synthesised by the decarboxylation of ornithine by the key enzyme ornithine decarboxylase (ODC, EC 4.1.1.17) [8]. Increased polyamine de novo synthesis is known to occur in rapidly proliferating cells including tumour cells [9]. Therefore, polyamine metabolism via the control of ODC protein activity and expression has for several years been considered as a target for chemotherapy [9–11]. An alternate

Abbreviations: ADC, arginine decarboxylase; BrdU, bromodeoxyuridine; DAO, diamine oxidase; DFMO, difluoromethylornithine; DMEM, Dulbecco modified Eagle medium; CDKs, cyclin-dependent kinases; CDKIs, cyclin-dependent-kinases inhibitors; FCS, fetal calf serum; HS, horse serum; LDH, lactate dehydrogenase; ODC, ornithine decarboxylase; PCA, perchloric acid; PI, propidium iodide; SAMDC, *S*-adenosylmethionine decarboxylase; SSAT, spermidine/spermine N1-acteyltransferase; DENSPM, N1, N11-diethylnorspermine; N1A-Spd, N1-acetylspermidine; Put, Putrescine; Spd, spermidine; Spm, spermine; Agm, agmatine

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pathway to putrescine biosynthesis from L-arginine is well known to occur in bacteria and plants but is also found in mammals [12–15]. In mammals, it occurs via the direct decarboxylation of arginine through arginine decarboxylase (ADC EC 4.1.1.19) to produce agmatine, which is then converted into putrescine by agmatinase in different tissues including the brain, liver and kidney [12,16–19].

Agmatine is present in the lumen of the human gastrointestinal tract and stems from three sources [12]: (a) agmatine formed and released by colonic flora [5], (b) unabsorbed agmatine contained in variable amounts in ingested food [4,12,20], (c) luminal agmatine derived from desquamated intestinal epithelial cells. Among its numerous physiological roles [21–24], agmatine has been shown to interfere with polyamine metabolism using ex vivo and in vitro experiments [25–28] and with cell growth in non-intestinal tumour and healthy cells without exerting any toxic effects [25,29–32]. Moreover, exogenous agmatine is known to be absorbed by the gastrointestinal tract and strongly accumulated in the liver and intestine [33].

Thus, the aim of the present study was to document whether agmatine altered cell proliferation in two human colon adenocarcinoma epithelial cell lines, namely HT-29 and $CaCo_2$. Then, we characterised the agmatine effect on polyamine metabolism and on cell cycle distribution, DNA synthesis and cell cycle-regulated protein expression. In addition, we determined that agmatine strongly accumulated within colonic cells without being metabolised.

2. Materials and methods

2.1. Materials

Agmatine sulfate, which was purchased from Fluka (St Quentin Fallavier, France), contained no putrescine and only 1% spermine. Methylbenzethonium hydroxide was purchased from Fluka. L-Ornithine hydrochloride, bis-benzymide trihydrochloride (HOECHST33342), pyridoxal 5′-phosphate, and dithiotreitol were purchased from Sigma chemicals (St Quentin Fallavier, France). A protease inhibitor complete mini cocktail was purchased from Roche Diagnostics GmbH (Mannheim, Germany). L-[1-^{14}C] ornithine, [1,4-^{14}C] putrescine, L-[guanido-^{14}C] arginine and L-[1-^{14}C] glucose were purchased from New England Nuclear (Boston, MA, USA). [Guanido-^{14}C]agmatine was purchased from Bio Trend (Köln, Germany).

2.2. Cells and culture conditions

The HT-29 $Glc^{-/+}$ cells used in this study were kindly provided by Zweibaum et al. [34]. These human colon adenocarcinoma cells were selected from the parental line by growing them in a glucose-free medium for 36 passages and then switching them to a 25 mM D-glucose containing medium. These cells are known to accumulate and metabolise butyrate, which is the major energetic substrate of healthy colonic epithelial cells [35]. HT-29 $Glc^{-/+}$ cells were grown at 37 °C under 10% CO_2 atmosphere in a Dulbecco modified Eagle medium (DMEM) containing 4 mM L-glutamine, 25 mM D-glucose and supplemented with 10% fetal calf serum (FCS). $CaCo_2$ cells were cultured in the same medium except for the presence of 20% FCS and 1% non-essential amino acids. HT-29 $Glc^{-/+}$ cells were used from passage 40 to 71 and $CaCo_2$ cells were used from passage 101 to 127 (1 passage per week). Agmatine sulfate or equal amounts of H_2SO_4 (control) was always added 2 days after cell seeding in DMEM supplemented with 3% horse serum (HS) in order to avoid the polyamine degradation by serum amine and diamine oxidase (DAO) activities. Indeed, DAO is involved in agmatine and polyamine catabolism. Using the Okuyama and Kobayashi method [36], we found that horse serum contained less DAO activity than FCS (0.37 ± 0.11 pmol/μl/h versus 0.88 ± 0.10 pmol/μl/h) so that the diamine oxydase present in the cultured medium supplemented with 3% HS was reduced eight fold in comparison with the culture medium containing 10% FCS. H_2SO_4 controls were first maintained under 10% CO_2 atmosphere in order to reach a neutral pH before the experiments. To test the long-term effect on cell proliferation, the media were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml amphotericin B. The media were changed every day.

2.2.1. Cell proliferation

HT-29 $Glc^{-/+}$ and $CaCo_2$ cells were seeded at a density of 5×10^3 cells per well on 96-well tissue culture microplates (Costar, Marne la Vallée, France) and cell proliferation was determined using the method of DNA fluorometric assay using bis-benzimide trihydrochloride i.e. Hoechst 33342 [37]. Fluorescence intensity was quantified (λex=360 nm, λem=465 nm) using the microplate cytofluorometer Spectrafluorplus (TECAN, Trappes, France). The results were expressed as the number of cells per well using a linear standard curve.

2.2.2. Membrane integrity

Membrane integrity was estimated by the release of the cytosolic enzyme, lactate dehydrogenase (LDH), in the culture medium [38,39]. Briefly, after agmatine treatment, the culture medium and adhering cells recovered after trypsinization were used to measure LDH activity. Membrane integrity was calculated as the ratio of LDH activity in adhering cells versus total LDH activity (the sum of both fractions).

2.3. Determination of intracellular polyamine content and putrescine biosynthesis via ODC

HT-29 cells were cultured with 2 mM agmatine sulfate or 2 mM H_2SO_4 into DMEM containing 3% HS. Twenty-four hours later, the cells were harvested and the cell pellets were

washed with PBS, centrifuged and kept at −80 °C until analysis. The cell pellets were homogenised in perchloric acid (PCA, 200 mM) and the homogenates were centrifuged at 3000×*g* for 10 min after standing 16 h at 2 °C. The acid insoluble pellets were used for protein determination and the clear supernatants were applied on a reversed-phase C18 column for separation. Agmatine and the polyamines were separated as ion pairs formed with *n*-octanesulfonic acid, and the column effluent was reacted with *o*-phtalaldehyde/2-mercaptoethanol reagent before monitoring fluorescence intensity [40]. Polyamine intracellular concentrations was estimated using HT-29 cell protein content (i.e. 213±13 μg proteins/10^6 cells) and the measured intracellular volume (i.e. 0.42 μl/10^6 cells, see Section 2.4). Putrescine biosynthesis measured as the rate of L-ornithine decarboxylation in H_2SO_4 or agmatine-treated isolated cells was measured according to the method previously described by Robert et al. [39].

2.4. Agmatine accumulation and metabolism

The accumulation and binding of radioactive agmatine by HT-29 cells was carried out by cell centrifugation through an oil layer [41]. Briefly, 2 days after seeding, the isolated cells (0.3×10^6) were incubated for 90 min at 37 °C or 4 °C in 120 μl incubation medium containing 1 mM [guanido-^{14}C]agmatine and were then centrifuged (12 000 g, 5 min) through a silicon oil layer (Nyosil M2O, NYE lubricants, New Bedford, MA) and recovered in 50 μl of an aqueous solution containing 0.64 M CsCl and 0.05 M HCl. The radioactivity of the cell pellet was measured by liquid scintillation. In all experiments, the net uptake of agmatine was corrected for the extracellular contamination space using 1 mM L-[1-^{14}C] glucose incubated with cells at 4 °C (i.e. 0.15±0.04 μl/10^6 cells, n=4). The amount of agmatine bound to the extracellular cell surface was measured at 4 °C after correction for the extracellular contamination. The agmatine accumulation within the cells was obtained by the difference between the results obtained at 37 °C and 4 °C. The intracellular volume of the HT-29 cells was estimated by calculating the difference between L-glucose spaces measured at 37 °C and 4 °C (i.e. 0.42±0.08 μl/10^6 cells, n=4) as previously described [35].

The flux of agmatine through agmatinase was estimated by measuring the radioactive urea production from [guanido-^{14}C]agmatine in the absence or presence of the DAO inhibitor aminoguanidine (1 mM). Two days after seeding, isolated cells were incubated for 90 min at 37 °C in 120 μl incubation medium (see above) containing 1 mM [guanido-^{14}C]agmatine. After the addition of PCA (2% final concentration), urea was separated from agmatine on a partisphere C18 column (Whatman, Clifton, NJ) using buffers described by Seiler and Knodgen [40] and the previously described gradient [42].

De novo agmatine and urea biosynthesis via ADC and arginase respectively were measured by agmatine and urea productions from radioactive L-arginine in cells isolated 2 days after seeding. The cells were incubated for 90 min at 37 °C in 120 μl incubation medium containing 0.4 mM L-[guanido-^{14}C] arginine. Urea was separated from agmatine on a partisphere C18 column as described above.

2.5. Immunoblot analyses of ODC protein and cell cycle-related proteins

HT-29 cells were cultured without or with agmatine sulfate or H_2SO_4 for 24 h at the doses of 1 mM for the ODC protein determination or 5 mM for cell cycle-related protein analyses. The cells were washed twice with PBS, lysed on ice and scraped using a Tris–HCl buffer (50 mM Tris–HCl pH 7.5, 10 mM DTT, 0.1 mM EDTA 0.5% NonodetP40) containing protease inhibitors. Homogenates were then sonicated on ice for 3×10 s. The samples were centrifuged at 10 000×*g* for 10 min at 4 °C and protein concentrations were determined using the Lowry procedure. One hundred micrograms of protein was then separated on 7.5% SDS-PAGE for the determination of ODC protein expression while 15 μg of protein was separated on 12% SDS-PAGE for cell cycle proteins. Following electrophoresis, the proteins were transferred onto nitrocellulose membranes (Amersham Pharmacia biotech, Orsay, France) at 20 V overnight at 4 °C.

The membranes were probed with one of the following appropriately diluted primary antibodies: anti-ODC (Sigma, 1:4000), anti-p27 ^{kip1} (BD Biosciences, 13231A, 1:1000), anti-cyclin B1 (Upstate biotechnology, Lakeplacid, NY, 05-373, 1:2000); Santa Cruz Biotechnology antibodies (Santa Cruz, 1:1000): anti-CDK1 (H-297), anti-CDK2 (M2), anti-CDK4 (C-22), anti-CDK6 (C-21), anti-cyclin D1(R-124), anti-cyclin A (H-432), anti-cyclin E (M20), anti-p21$^{waf1/cip1}$ (C-19), anti-p57^{kip2} (C-20). This was then followed by incubation with the corresponding peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Protein bands were visualised using the ECL+ kit (Amersham Biosciences, Saclay, France). To verify loading homogeneity, the membranes were re-incubated with anti-α tubulin antibody (1:20 000, Sigma) followed by anti-mouse peroxidase-conjugated antibody. Protein bands were quantified by densitometry of the film using a Las1000 camera (Fujifilm, Paris, France) and Aida software (Raytest, Strasbourg, France).

To visualise the activation peak of ODC during the cell cycle, ODC expression along the different cell cycle phases was checked using HT-29 cells synchronised at the G_1 and S phases [43]. Briefly, 2 days after seeding, HT-29 cells were synchronised in the G_1 phase by incubating cells in a serum-free medium for 24 h. The cells were synchronised at the S phase by treating G_1 phase cells with 5 μg/ml aphidicholin (Sigma) in 10% FCS medium for 24 h followed by washing and culture of the cells in the drug-free 10% FCS medium for 3 h. Cell synchronisation was controlled by flow cytometry as described below.

2.6. Cell cycle distribution

Preliminary experiments indicated that the maximal G_1 phase accumulation without any noticeable apoptosis was obtained after 24 h of serum free culture conditions (G_0/G_1, 88.4% ; S, 10.2%; G_2/M, 1.5%). Cells synchronised in the G_1 phase were cultured for different periods of time in the presence of 5 mM agmatine sulfate, 5 mM H_2SO_4 or control medium containing 3% HS.

Cells ($1–2\times10^6$) were recovered at different times following an exposition to 5 mM agmatine sulfate or 5 mM H_2SO_4, centrifuged at $500\times g$ for 5 min and the cell pellets were washed twice with phosphate buffer saline. The cells were fixed and stained with propidium iodide (PI) solution containing ribonuclease, using the Nuclear Isolation Medium (NIM: 100 ml PBS; 10 mg deoxyribonuclease-free ribonuclease; 100 μl Triton ×100; and 5 mg PI). Flow cytometry analyses were performed on a FACScan (BD Biosciences, Le Pont de Claix, France). For each time point, 20×10^3 cells were analysed and PI fluorescence was displayed on a linear scale using the FL2 photodetector. The DNA profile indicates the relative abundance of G_O/G_1, S and G_2/M phase populations. Cell distribution into cell cycle phases were calculated using the ModFit flow cytometry modelling (V 3.0) software.

2.7. BrdU-immunoflurescent labelling and flow cytometry analyses

To measure DNA synthesis, G_1-arrested HT-29 cells were pulse-labelled for 1 h with 10 μM bromodeoxyuridine (BrdU) using the BrdU Flow kit (BD Biosciences) at 37 °C, 6, 9, 12 or 23 h after the restart of the culture with or without agmatine 5 mM. Then the cells were either harvested immediately after the BrdU-labelling period or after a period of post-labelling incubation (until 24 h total culture). Cell fixation, DNA denaturation, anti-BrdU-FITC and 7-Amino-Actinomycin D (7AAD) staining and flow cytometry were then conducted. One million cells were recovered, fixed and permeabilised using the Cytofix/Cytoperm kit according to the manufacturer's protocol. To expose incorporated BrdU, the cells were then treated with DNase (30 μg DNase/10^6 cells) for 1 h at 37 °C. Staining of BrdU-containing DNA was performed using 50 μl of anti-BrdU-FITC antibody (1/200, BrdU Flow Kit). Then staining of total DNA was made using 20 μl of 7-AAD solution (Via probe, BD Biosciences). The cell samples were then analysed for BrdU content (FITC) and total DNA content (7-AAD) using an EPICS XL4C flow cytometer (Beckman Coulter, Villepinte, France).

2.8. Data analysis

The results were expressed as means (±S.E.) of independent experiments performed with the cells isolated at different passages. Statistical significance was assessed using one way ANOVA followed by pairwise multiple comparison procedures.

3. Results

3.1. Agmatine reduces cell proliferation without altering membrane integrity

The effect of agmatine on HT-29 cell proliferation is reported in Fig. 1. Agmatine markedly inhibited HT-29 cell growth in a dose-related manner. This effect was apparent from the concentration of 1 mM. The agmatine IC50 (concentration inhibiting cell growth by 50%) was approximately 2 mM and an almost complete inhibition of cell growth was evident at 5 mM of agmatine (Fig. 1). To test whether agmatine was cytotoxic for HT-29 cells, cell viability was assessed by measuring the release of cytosolic lactate dehydrogenase. At the highest concentration (5 mM), agmatine did not affect the percentages of living cells after a 24 h treatment (97.4±0.3%, n=6 versus control 97.3±0.5%, n=8) or after a 120 h treatment (92.6±0.5%, n=5, versus control 96.2±1.4%, n=6). Finally, to ensure that this cytostatic effect of agmatine was not restricted to HT-29 cells, we tested it on $CaCo_2$ cells. Agmatine also inhibited $CaCo_2$ cell proliferation similarly to HT-29 cells (same IC50 of agmatine for both cell lines) and did not alter membrane integrity (data not shown).

These results show that agmatine strongly inhibits epithelial colonic malignant cell growth without altering cell membrane integrity.

3.2. Agmatine alters the polyamine content of HT-29 cells

Since polyamines are essential for cell proliferation, we measured the effect of agmatine on "classical" polyamine contents (Table 1). Agmatine was absent from untreated

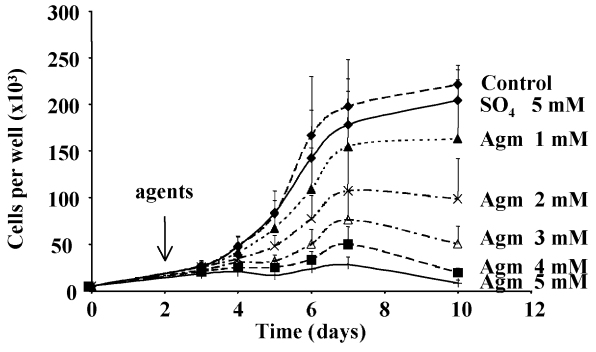


Fig. 1. Effect of agmatine on HT-29 cell growth. Two days after seeding, HT-29 cells were cultured in the absence or presence of increasing concentrations of agmatine sulfate (Agm, 1 to 5 mM) or 5 mM H_2SO_4 (SO_4). The media were changed every day. The number of cells was then measured using the Hoechst DNA fluorometric assay. The results are expressed as mean values (±S.E.) of 4 independent experiments carried out in multicate.

Table 1
Intracellular contents of polyamines in HT-29 cells

	Agmatine	Put	N_1A-Spd	Spd	Spm
	pmol/mg protein				
Added agents					
None	0	293±26	0	1703±84	5204±263
H_2SO_4 2 mM	0	396±9**	0	1933±40	4862±65
Agmatine sulfate 2 mM	24 000±839***	71±5***	33±4*	320±21***	5159±106

Two days after seeding, HT-29 cells were cultured for 24 h without or with the indicated agents. Cells were isolated and rinsed with PBS and cell pellet polyamine contents were determined as described in Materials and methods.
Values represented mean±S.E. from four independent experiments.
* $P<0.05$, the ANOVA analysis.
** $P<0.01$, the ANOVA analysis.
*** $P<0.001$, the ANOVA analysis.

control cells. At the concentration that reduced the basal cell growth by 50% (i.e. 2 mM), agmatine massively accumulated within the cells after a 24 h treatment and reduced putrescine and spermidine concentrations while it increased the N_1-acetylspermidine intracellular content. The spermine cellular pool was unaffected. Similar results were observed with 5 mM agmatine (data not shown). The presence of N_1-acetylspermidine in agmatine-treated cells indicates that agmatine up-regulated the polyamine catabolic pathway.

In summary, as previously shown in other cell lines, agmatine decreases intracellular levels of putrescine and spermidine in HT-29 cells and apparently also increases their catabolic pathway.

3.3. Agmatine reduces the rate of L-ornithine decarboxylation and ODC protein expression

Since ODC is the first and rate-limiting enzyme in polyamine synthesis, we sought to elucidate whether agmatine interferes with polyamine metabolism in colonic cells. Thus, we measured the rate of L-ornithine decarboxylation via ODC in agmatine-treated HT-29 cells (Fig. 2). At 1 mM, the flux of L-ornithine through ODC was reduced by 60% after 1 h and by 95% after 4 h or more, indicating that agmatine markedly lowers the polyamine biosynthetic capacity of these cells. Similar results were observed with $CaCo_2$ cells (data not shown).

A number of studies have shown that ODC activity and protein expression is modulated during the cell cycle in different cell types. In fact, the ODC plays an important role in the G_1/S transition in the cell cycle [6,43]. Thus, to determine whether the agmatine-reduced polyamine biosynthetic capacity was due to an alteration of the ODC protein level, immunoblotting was performed (Fig. 3). As compared with untreated cells, ODC protein level decreased by 70% (n=4, P=0.029) within 24 h in the presence of 1 mM agmatine. Moreover, as previously described by Chen et al. [43] we confirmed that ODC protein expression peaks in S phase-synchronised HT-29 cells, and remains low in the G_1 phase-arrested cells.

These results indicate that agmatine reduces polyamine biosynthesis and cell content in HT-29 cells mainly by decreasing the amount of ODC protein whose expression is maximal in the S-phase.

3.4. Agmatine metabolism in HT-29 cells

Different experiments were carried out to characterise agmatine biosynthesis and catabolism in HT-29 cells. The accumulation of agmatine was determined by measuring the uptake of [guanido-^{14}C]agmatine in the cells (Table 2). Using the measured intracellular volume (i.e. 0.42±0.08 μl/10^6 cells) for HT-29 cells, we calculated that after a 90 min incubation in the presence of 1.0 mM [guanido-^{14}C]agmatine, the intracellular concentration of agmatine averaged 6.3 mM. Compared to net accumulation, less than 10% of agmatine was bound to the cell surface (Table 2).

HT-29 cells were found to have a very low capacity for agmatine synthesis via ADC (Table 2). The lack of agmatine formation was not due to L-arginine catabolism via another catabolic pathway. Indeed, 20 mM L-valine, which inhibited

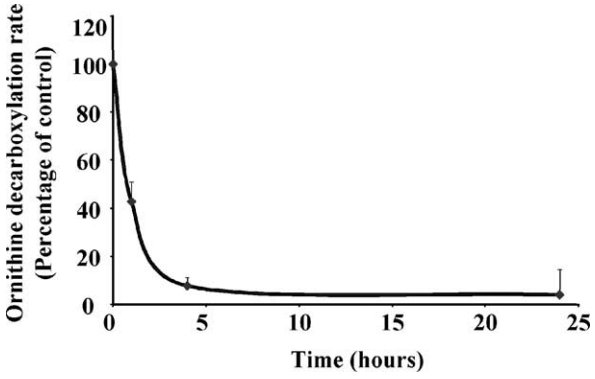


Fig. 2. Effect of agmatine on the rate of L-ornithine decarboxylation in HT-29 cells. Two days after seeding, 1 mM of agmatine sulfate was added to the culture medium for 1, 4 or 24 h. L-Ornithine decarboxylation was then determined in isolated HT-29 in the presence of 1 mM L- [1-^{14}C] ornithine at 37 °C and for 90 min in the presence or absence of 10 mM DFMO, known to completely inhibit ODC activity. The DFMO-sensitive production of radioactive CO_2 was measured. Ornithine decarboxylation rates (mean±S.E.) are expressed as percentages of the control value (1 mM H_2SO_4) and represent 3 independent experiments carried out in duplicate. The basal L-ornithine decarboxylation rate averaged 2452±334 pmol/10^6 cells/90 min (n=14).

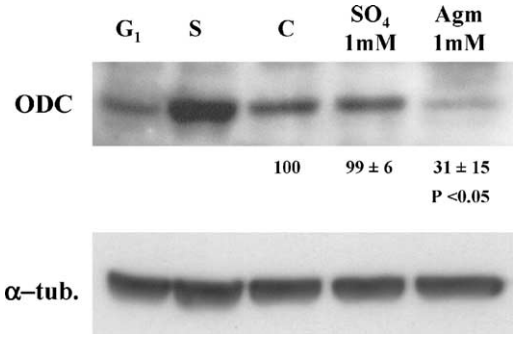


Fig. 3. Effect of agmatine on the ODC protein expression in HT-29 cells. HT-29 cells were cultured in the presence of 1 mM agmatine sulfate (Agm) or 1 mM H_2SO_4 (SO_4) for 24 h. Proteins (100 μg/lane) were loaded and probed with monoclonal anti-ODC antibody as described in the Material and methods. ODC protein expression was also measured at the G_1 and S phases in synchronised HT-29 cells in order to confirm ODC over-expression during the S phase. This result represents one experiment representative of four independent experiments. The number above corresponds to the mean±S.E. relative density of the spots measured by image analysis. α-Tubulin was used as the control for protein loading.

the basal flux of 0.4 mM L-[guanido-^{14}C] arginine through arginase (i.e. 84±11 pmole/10^6 cells/90 min, n=4) by 66%, did not increase agmatine production from L-arginine (data not shown).

Agmatinase-catalysed agmatine catabolism (which forms putrescine and urea) was next determined by measuring radioactive urea production from 1 mM [guanido-^{14}C]agmatine. Only small amounts of urea were produced by HT-29 cells (Table 2), indicating that agmatine catabolism in these cells is very limited. Since radioactive agmatine can also be used as a substrate by DAO, γ-guanidinobutyraldehyde dehydrogenase and γ-guanidobutyrate hydrolase [44], experiments were also carried out in the presence of 1 mM aminoguanidine, a specific DAO inhibitor. Urea production in the presence of this inhibitor (i.e. 41±13 pmol/10^6 cells/90 min, n=4) was not significantly different from the results obtained in its absence (Table 2), indicating that agmatine catabolism in HT-29 cells mainly occurs through agmatinase. Treatment of the cells for 24 h with 1 mM agmatine did not modify the fluxes of L-[guanido-^{14}C]arginine through ADC, arginase or agmatinase (data not shown).

In summary, our results indicate that HT-29 cells do not synthesise de novo agmatine and that agmatine catabolism via agmatinase by these cells is of minor importance.

3.5. Agmatine affects the HT-29 cell cycle distribution

Polyamine content is regulated during the cell cycle and a number of studies have shown an association between cell cycle arrest and a disruption of polyamine biosynthesis. We therefore next investigated the effect of agmatine on cell cycle repartition in freely cycling growing cells. Since maximal growth inhibition was observed using 5 mM agmatine in colon carcinoma cells (see above), we used this concentration for our study. The cells treated with 5 mM agmatine for 24 h showed a significant increase in the number of cells in the S and G_2/M phases and a decrease in the G_1 phase when compared to the control experiments (Table 3). Similar results were obtained with $CaCo_2$ cells (data not shown). No sub-diploid peak was observed in agmatine-treated cells (data not shown). The agmatine-induced-modification in HT-29 cell cycle distribution was also evident after a 72 h treatment (Table 3) and 120 h treatment (data not shown). These results indicate that agmatine alters the cell cycle in colonic tumour cells.

We further investigated the kinetics of 5 mM agmatine effects on the cell cycle of serum-starved synchronised HT-29 cells (Fig. 4). We showed that after 12 h, the cells treated with agmatine were more frequently in the S phase and less in the G_1 phase than control cells (P<0.05). From 24 h to 48 h, the agmatine effect was even more pronounced and the cells were found to accumulate both in the S and G_2/M phases at the expense of the G_1 phase (P<0.01).

Table 2
Agmatine metabolism in HT-29 cells

Measured parameters	pmol/10^6 cells/90 min
Net accumulation of agmatine	2645±311
Binding of agmatine	240±56
Production of radioactive urea from agmatine via agmatinase	59±18
Production of radioactive agmatine from L-arginine via arginine decarboxylase	3±1

Two days after seeding, HT-29 cells were isolated and incubated for 90 min at 37 °C or at 4 °C in the presence of 1 mM [guanido-^{14}C]agmatine. The 1 mM [guanido-^{14}C]agmatine uptake recorded at both temperatures were corrected using the L-[1-^{14}C]glucose space measured at 4 °C. The binding of agmatine to the outer side of the cells (measured at 4 °C) was subtracted from the uptake measured at 37 °C to calculate the agmatine net accumulation into cells. Cell productions of urea from 1 mM L-[guanido-^{14}C]agmatine and agmatine production from 0.4 mM L-[guanido-^{14}C] L-arginine were measured after 90 min incubation at 37 °C by HPLC. Data are expressed as mean values (±S.E.) from four independent experiments.

Table 3
Agmatine affects the HT-29 cell cycle distribution

Added agents	Cell cycle phases (%)		
	G0/G1	S	G2/M
24-h treatment			
Control	64.4±2.4	26.7±1.8	8.9±1.1
H_2SO_4 5 mM	65.0±2.4	25.8±1.7	9.1±1.0
Agmatine sulfate 5 mM	45.1±2.8***	39.0±2.0***	15.9±1.2**
72-h treatment			
Control	71.2±2.7	20.7±1.5	8.1±1.6
H_2SO_4 5 mM	70.3±2.8	21.1±1.6	8.5±1.5
Agmatine sulfate 5 mM	55.7±2.8**	28.5±1.7**	15.8±1.3**

HT-29 were cultured in the absence or in the presence of 5 mM agmatine for 24 or 72 h and then analysed for cell cycle distribution using flow cytometry after staining by propidium iodide. Results are provided as percentage of total cells and represent mean values±S.E. of three to six independent experiments.

** P<0.01, the ANOVA analysis.

*** P<0.001, the ANOVA analysis.

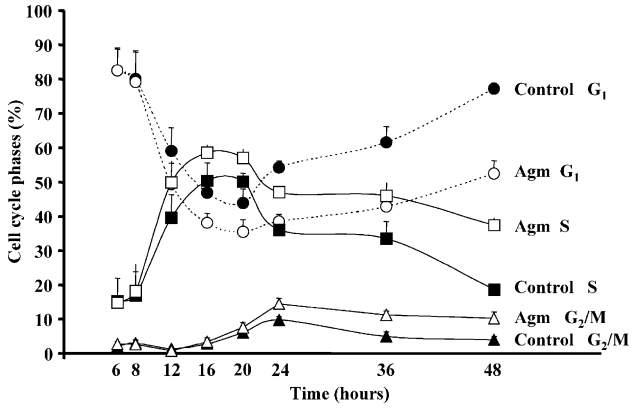


Fig. 4. Kinetics of agmatine effects on the cell cycle of G_1-arrested HT-29 cells. HT-29 cells were synchronised in the G_1 phase after 24 h culture in serum-free medium. They were then cultured in the absence (control) or in the presence of 5 mM agmatine sulfate (Agm) from period of time ranging from 6 to 48 h. The cell cycle distribution was analysed using flow cytometry after staining with propidium iodide. The results are provided as percentages of total cells and represent mean values±S.E. of four independent experiments. Plain symbols were used for control cells and empty ones for agmatine-treated cells.

These results indicate that there is an accumulation of cells in the S phase and then in the S and G_2/M phases after 12 to 48 h of agmatine treatment.

3.6. Agmatine reduces cyclin A and cyclin B_1 protein expression in HT-29 cells

Transition events in the cell cycle are controlled by phosphorylations carried out by cyclin-dependent kinases (CDKs) which are controlled by their activators (cyclins) or inhibitors (CKIs). Since agmatine alters the cell cycle distribution in HT-29 cells, we next investigated the ability of agmatine sulfate (5 mM) to modulate the expression of these cell cycle regulatory proteins after 24 h (Fig. 5). By comparing the control (no agent added), we confirmed that H_2SO_4 treated control cells did not modify the expression of any cell cycle regulatory proteins (Fig. 5). In turn, agmatine sulfate (5 mM) reduced the level of cyclin A and cyclin B_1 proteins by 23% (Fig. 5) without altering the expression of the CDK1, CDK2, CDK4, CDK6, cyclin D1, and CKI tested ($p27^{kip1}$, $p57^{kip2}$). We also noted that $p21^{waf1/cip1}$ (Fig. 5) and cyclin E (data not shown) were undetectable at 24 h. The results of dose-dependent experiments using 1, 2 and 5 mM of agmatine indicated that only 5 mM of agmatine significantly reduced the expression of cyclins A and B_1 (data not shown).

Since we observed that agmatine first increased the S phase proportion in HT-29 from 12 h incubation followed by an accumulation of HT-29 in the S and G_2/M phases, we further examined the agmatine effect on the time course of the expression of the S phase- and G_2/M phase-regulated proteins: cyclin A, cyclin B_1 and $p21^{waf1/cip1}$ (Fig. 6). In the control and H_2SO_4 treated cells, the levels of cyclins A and B_1 reached a maximum after 24 h incubation then declined after 36 h. In the 5 mM agmatine-treated cells, levels of cyclins A and B_1 were lower after 24 h culture and then increased after 36 h in contrast to what was observed in the control cells, indicating a possible delay of cyclin expression. The p21 protein level increased rapidly, reached a maximum after 6 h of incubation, then decreased to an undetectable level. Agmatine did not significantly alter p21 expression in HT-29 cells (Fig. 6).

In summary, in HT-29 cells, agmatine reduced the amounts of cyclins A and B_1 after 24 h and increased them

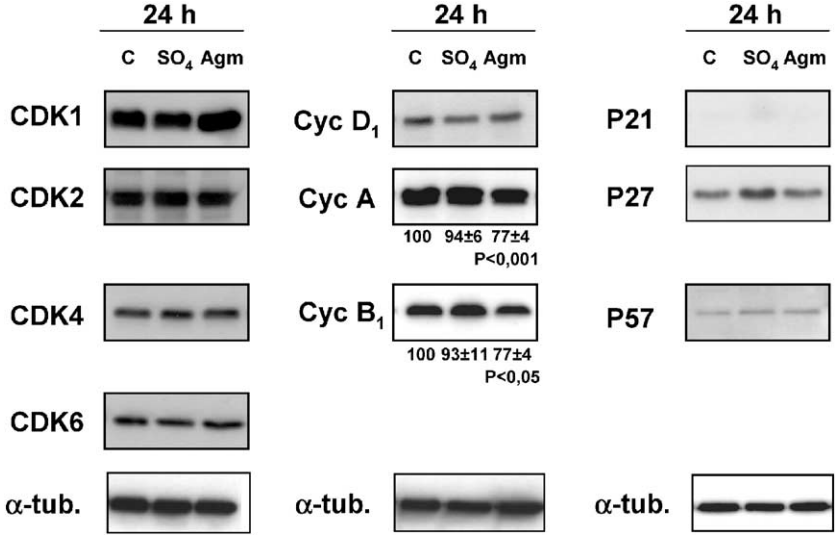


Fig. 5. Expression of cell cycle proteins in HT-29 cells treated with agmatine. HT-29 cells were synchronised in G1 after 24 h culture in serum-free medium. The cells were then cultured without (C) or with either 5 mM agmatine sulfate (Agm) or 5 mM H_2SO_4 (SO_4) for 24 h and whole cell lysates were prepared. Membranes were probed for CDKs, cyclins and the CIP family of CKIs. α-Tubulin was used as the loading control. Representative blots of at least three separate experiments are shown.

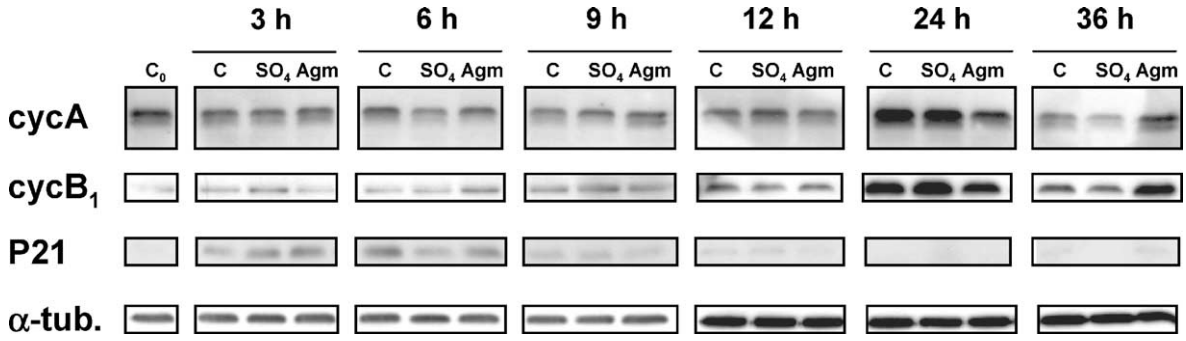


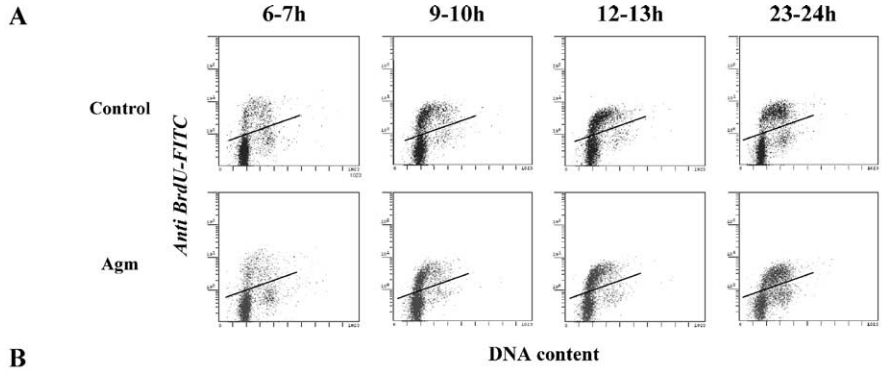
Fig. 6. Kinetics of agmatine effects on the expression of cyclin A, cyclin B_1 and P21 in HT-29 cells. HT-29 cells were synchronised in G_1 after 24 h culture in serum-free medium. The cells were then cultured without (C) or with either 5 mM agmatine sulfate (Agm) or 5 mM H_2SO_4 (SO_4) from period of time ranging from 3 to 36 h. Membranes were probed for cyclin A, cyclin B_1 and p21. α-Tubulin was used as the loading control. Representative blots of at least two separate experiments are shown.

after 36 h incubation, suggesting that agmatine delays their expression during the cell cycle.

3.7. Agmatine affects DNA synthesis in HT-29 cells

Since agmatine-treated HT-29 cells compared to the control showed different proportions in the S phase and different expression of cyclin A and B_1, these cells were used to explore the role of agmatine on the rate of DNA synthesis using BrdU incorporation experiments (Fig. 7A and B).

BrdU analysis showed that, for the first two periods of labelling, neither the labelling index (LI) nor the percentage of G_1/S BrdU-positive cells changed significantly between treatments (Fig. 7A and B). In contrast, from the period 12–13 h and thereafter, the LI and the percentage of G_1/S labelled cells increased with agmatine, suggesting that



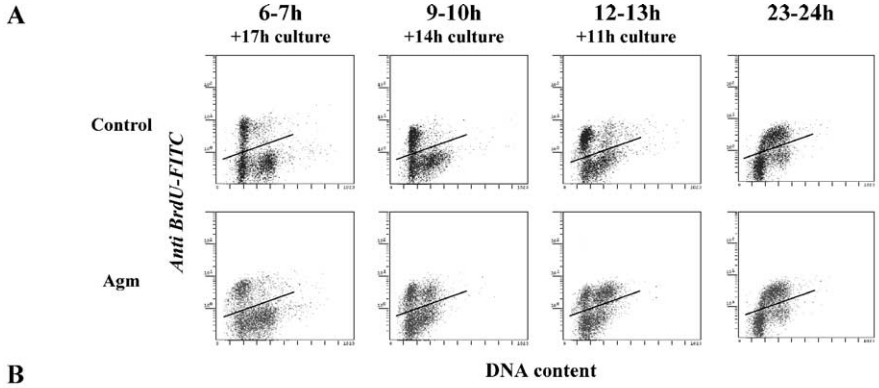
	Treatment	6-7h	9-10h	12-13h	23-24h
LI= % total BrdU-incorporating cells [a]	Control	12.2	18.1	30.0	23.7
	Agm	12.0	16.9	36.4	34.8
% BrdU-incorporating cells in G_1/S [b]	Control	5.1	13.0	19.0	6.4
	Agm	6.5	11.2	23.7	9.5
Mean FITC [c]	Control	3.47	2.93	3.04	4.84
	Agm	3.22	2.21	2.72	4.40
Relative DNA synthesis [d]	Control	42.3	53.0	91.2	114.7
	Agm	38.6	35.8	99.0	153.1

Fig. 7. Effect of agmatine on DNA synthesis in HT-29 cells. (A) G_1-arrested HT-29 cells were pulse-labelled with 10 μM BrdU at 37 °C for 1 h at the beginning of the 6, 9, 12 or 23 h periods after the restart of the culture containing or not 5 mM agmatine sulfate, followed by fixation, staining with anti-BrdU-FITC and 7-AAD and flow cytometry analyses. Cell cycle analyses were carried out using two-parameter flow cytometry. The amount of BrdU incorporation per cell is expressed on the *Y* axis (anti-BrdU-FITC), whereas the DNA content per cell is expressed on the *X* axis. BrdU-labelling cells are localised above the line. (B) The data reported here correspond to one representative experiment from 4 independent analyses. [a]LI (labelling index): data corresponded to total BrdU-labelled cells localised above the line in the two parameter flow cytometry parameter window (Panel A). [b]Data were BrdU-positive cells corresponding to the G_1/S subpopulation containing G_1DNA (Panel A). [c]Data represented mean FITC value of total BrdU-positive cells. [d]Relative DNA synthesis in all BrdU-labelled cells was calculated by multiplying the mean FITC value by the number (percentage) of all BrdU-labelled cells.

agmatine-treated cells were delayed at the G_1/S boundary or early S phase (Fig. 7A). The percentage of labelled cells was the consequence of G_1/S and S cell accumulation (Fig. 7B). The S fraction, ranging from 36% of the BrdU-positive cells, was unchanged by agmatine for the 12–13 h period, while it was increased by agmatine for the last labelling period (ranging from 47% to 72%). The mean FITC signal, which reflects the DNA synthesis rate per cell, was always reduced by the agmatine treatment, indicating that agmatine lowered the DNA synthesis rate. However, since the total amount of DNA synthesised calculated for all the labelled cells (Fig. 7B) depends on the number of labelled cells in the S phase, it was slightly reduced by agmatine for the first two time points and markedly increased for both of the last periods (Fig. 7B).

Using the BrdU-labelling method followed by post-labelling culture which corresponds to labelled cells that continue to progress through the cell cycle, we studied the effect of 5 mM agmatine on the length of the S phase (Fig. 8A and B). The progression of BrdU-labelled cells from the S phase through G_2/M and into G_1 was followed by determining the percentage of BrdU-positive cells present in the G_1 phase (Fig. 8B). When cells were labelled at the period (6–7 h), more than 75% of the positive cells were found in the G1 phase after the additional 17 h culture. Their progression did not seem to be affected by the agmatine treatment (Fig. 8A). In turn, when control cells were labelled at the later periods of time (9–10 h and 12–13 h), more than 80% of the BrdU-positive cells were recovered in the G_1 phase. For the same labelling periods, only 72% and 46% of positive agmatine-treated cells were found in the G_1 phase while 27% and 54% were still in the S phase. These results indicate that the S and G_2/M phases were prolonged in the agmatine-treated cells. As reported above, from the 12–13 h period and later, agmatine reduced the LI and decreased the mean FITC signal, indicating that agmatine diminishes the rate of DNA synthesis per cell (Fig. 8B).

In conclusion, we can say that agmatine reduces the rate of DNA synthesis per cell and prolongs both the DNA synthesis duration per cycle and the cell cycle length in HT-29 cells.



B

	Treatment	6-7h +17h culture	9-10h +14h culture	12-13h +11h culture	23-24h
LI= % total BrdU-incorporating cells [a]	Control	24.6	36.9	43.2	23.7
	Agm	24.6	30.0	35.9	34.8
% BrdU-incorporating cells in G_1 [b]	Control	19.5	32.3	37.0	6.4
	Agm	18.8	21.8	16.5	9.5
Mean FITC [c]	Control	4.04	4.01	5.15	4.84
	Agm	3.20	3.32	3.43	4.40
Relative DNA synthesis [d]	Control	99.4	147.9	222.5	114.7
	Agm	78.7	99.6	123.1	153.1

Fig. 8. Effect of agmatine on the cell cycle length in HT-29 cells. (A) G_1-arrested HT-29 cells were pulse-labelled with 10 μM BrdU at 37 °C for 1 h at the beginning of the 6, 9, 12 or 23 h periods and followed by culture until the end of the cell cycle in the presence or the absence of agmatine sulfate 5 mM. Fixation, staining with anti-BrdU-FITC and 7-AAD and flow cytometry analyses were then performed; the results are presented using two-parameter flow cytometry. The amount of BrdU incorporation per cell is expressed on the *Y* axis (anti-BrdU-FITC), whereas the DNA content per cell is expressed on the *X* axis. BrdU-labelling cells are localised above the line. (B) Data reported here correspond to one representative experiment from 4 independent analyses. [a]LI (labelling index): data corresponded to total BrdU-labelled cells localised above the line in the two-parameter flow cytometry parameter window (Panel A). [b]Data were BrdU-positive cells corresponding to the G_1 subpopulation (Panel A). [c]Data represented mean FITC value of total BrdU-positive cells. [d]Relative DNA synthesis in all BrdU-labelled cells was calculated by multiplying the mean FITC value by the number (percentage) of all BrdU-labelled cells.

4. Discussion

Our study clearly demonstrates that millimolar concentrations of agmatine accumulated in colonic cells without being metabolised and reduced cell proliferation without exerting any cytotoxic effect. The growth-inhibitory effect of agmatine was concomitant with a rapid decrease in L-ornithine decarboxylation, reduced intracellular levels of putrescine and spermidine and, probably, increased polyamine catabolism. We have demonstrated that agmatine modified the progression of the cell cycle in HT-29 cells by accumulating the colonic cells in the S and G_2/M phases, by reducing the rate and the speed of DNA synthesis and by delaying cyclin A and cyclin B_1 expressions.

Agmatine reduced HT-29 and $CaCo_2$ cell growth with an IC50 of 2 mM. This value is in the range of the IC50 values previously measured using different intestinal tumour cell lines [45] and HeLa cells [29]. However, compared with other non-intestinal transformed or normal cells, i.e. MCT kidney cells, hepatocytes or astrocytes [25,31,32] where IC50 for agmatine ranged from 0.1 to 0.5 mM, HT-29 and $CaCo_2$ cells appear to be more resistant. This finding suggests that cell sensitivity to agmatine probably depends on the cell types.

We found that agmatine was not neosynthesised in HT-29 cells and was poorly catabolised. Only 2% of the accumulated agmatine was converted to urea and putrescine. The total of this 2% was converted through agmatinase rather than through DAO, the activity of which is close to the limit of detection in HT-29 cells [46]. These results are in accordance with the absence of ADC gene expression [47] and with the extremely low level of agmatinase mRNA expression in the colon [17]. Therefore, agmatine is most likely acting on the HT-29 cells by itself but not through metabolites generated through its cellular catabolism. The HT-29 cell capacity to accumulate agmatine is most probably a consequence of the presence in these cells of recently identified agmatine-specific transporters [48,49].

Putrescine, spermidine and spermine are required for cell growth and their intracellular levels are regulated by a complex equilibrium between synthesis via ODC and degradation driven by SSAT, as well as by cellular uptake and efflux [6,7,50]. The polyamine biosynthesis capacity is known to reach peaks during the cell cycle with ODC and SAMDC activities as well as cellular contents of putrescine, spermidine and spermine being high at the G_1/S and G_2/M transitions. ODC protein expression through the cell cycle is controlled primarily at the translation level following two independent mechanisms: a cap-dependent control during the G_1/S transition and a cap-independent mechanism during the G_2 or M phase [51]. As previously shown in different cell types [43,52–55], we confirmed that maximal ODC protein expression was observed during the early S phase in HT-29 cells and coincided with increased putrescine cellular content. As previously reported [45], we found that proliferative HT-29 cells contain micromolar concentrations of putrescine and millimolar concentrations of spermidine and spermine. In contrast, we did not detect agmatine in untreated HT-29 $Glc^{-/+}$ cells, a result which is consistent with the inability of these cells to synthesise agmatine via arginine decarboxylase (this study). In addition, as previously shown [25,26,56], we found that agmatine diminished putrescine and spermidine pools by reducing putrescine synthesis through a decrease in ODC protein expression. The increase in N_1-acetylspermidine observed in HT-29 cells treated with agmatine probably contributes to the decrease in the spermidine content. This fact suggests that SSAT activity in HT-29 cells is also up-regulated, as reported in different non-intestinal tissues [26,31,56]. Since ODC expression is down-regulated by agmatine in HT-29 cells (this study) and ODC activity is controlled during the cell cycle [6,7], we investigated the effect of agmatine on HT-29 cell cycle distribution, DNA synthesis and cell cycle-regulated protein expression. By contrast with data reported by Gardini et al. [56] which demonstrates a huge G_2/M increase and a marginal G_1 increase in rat hepatoma cells treated with agmatine for 72 h, we found that agmatine induced an accumulation of HT-29 cells in the S phase during the first cycle followed by an accumulation in the S and G_2/M phases at later time points. This discrepancy may be due to the different cell types and/or to the experimental conditions used. These results indicate that after entering the S phase, agmatine-treated HT-29 cells were inhibited in their progression through a reduction of the DNA synthesis rate and a prolongation of the time necessary for DNA synthesis. This situation was demonstrated by measuring the mean FITC signal and by observing the movement of BrdU-labelled cells through the entire cell population during the first cell cycle. This data was in agreement with those of Shie et al. [57] who reported that an inhibition of ODC expression was associated with a decrease in the DNA synthesis rate in HT-29 cells. In addition, CHO cells treated with a spermine analogue N1, N11-diethylnorspermine (DENSPN), which accumulated into cells, was shown to down-regulate polyamine biosynthesis and to up-regulate polyamine catabolism in HT-29 cells [58]. DENSPM-treated hamster ovary cells have a delay in the S phase during the first cycle after seeding [59]. In our study we found similar results with agmatine. We can, thus, propose that the nature and/or level of intracellular polyamine depletion via a combination of different modifications in polyamine metabolism may interfere with the cell cycle and DNA synthesis.

To further investigate the mechanisms responsible for the differences in cell cycle progression in agmatine-treated cells, we particularly focussed our attention on the expression of S and G2/M phase-regulatory proteins. We found that agmatine reduced cyclin A and B_1 protein expression without altering CDKs, cyclins D1, p21, p27 or

p57 expression. Although metabolic inhibitors of polyamine biosynthesis consistently reduce cell proliferation, their effects on cell cycle regulators differ considerably according to the drug used. For instance, DFMO, a suicide substrate analog of ODC, prevents in vitro cell growth by a G_1 arrest in IEC-6 and HT-29 intestinal epithelial cells, decreases the expression of CDK2, CDK4, cyclins D_1, D_2 and E proteins and increases p21 expression without altering cyclin A expression [60,61]. However, in such cells, DFMO, via ODC catalytic-specific inhibition, almost completely depleted the intracellular content of putrescine and spermidine and, to a lower extent, reduced the spermine content [61,62]. Thus, changes in intracellular concentrations of individual polyamines (i.e. agmatine, putrescine, spermidine, spermine and acetylated polyamines) and/or polyamine analogues may determine distinct effects on cell growth, cell cycle-regulator expression and DNA synthesis.

Polyamine analogues were shown to cause cell death or cell cycle arrest in relation to, at least in part, their ability to induce SSAT activity either on solid tumours or on cancer cells [63,64]. Indeed, causal relationships are difficult to establish with such analogues since they have numerous effects on polyamine metabolism. Moreover, conditional overexpression of SSAT in MCF7 human breast carcinoma cells is sufficient to inhibit cell growth with marked modifications in acetylated polyamine, putrescine and spermidine levels [64]. We thus hypothesise that agmatine, which accumulates in cells without being metabolised, is sufficiently similar to natural polyamines to interfere massively with polyamine metabolism but is not similar enough to functionally substitute for the missing natural polyamines for cell proliferation. This point reinforces the view that agmatine acts as a polyamine anti-metabolite [63]. Indeed, growing evidence indicates that the chemical nature as well as, possibly, the concentrations of the different polyamines or polyamine analogues present in cells can modify polyamine–DNA interactions and thus affect chromatin structure and DNA synthesis capacity [65].

In conclusion, agmatine, which is found in the human colon lumen [12], represents an interesting compound in relationship with colon carcinoma cells since agmatine interferes with both polyamine metabolism and DNA synthesis resulting in alterations in the cell cycle and cell growth. Further work is required to test in vivo the physiological relevance of our in vitro data and to evaluate the efficiency of agmatine from a nutritional chemopreventive perspective.

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