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Long-term treatment with Sitagliptin, a dipeptidyl peptidase-4 inhibitor, reduces colon carcinogenesis and reactive oxygen species in 1,2-dimethylhydrazine-induced rats

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Type 2 diabetes mellitus (T2DM) and insulin resistance (IR) increase colon cancer risk. Antidiabetic drugs stabilizing incretin hormones, such as inhibitors of dipeptidyl peptidase-4 activity (DPP4i), may affect colon carcinogenesis; however, the data remain controversial. Therefore, the authors studied whether long-term administration of the DPP4i Sitagliptin (SITA) affects 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis. Male F344 rats fed a high-fat (HF) diet promoting colon carcinogenesis and IR, were induced with DMH (100 mg/kg × 2 times). One week later, the animals were allocated to two groups: one continuing with HF diet (controls; $n = 8$) and one receiving SITA ($n = 8$) mixed in the diet (260 ppm). Body weight, food consumption and glycemia were not affected by SITA. Fifteen weeks after DMH, the number of the precancerous lesions mucin-depleted foci (MDF) was significantly lower in rats treated with SITA [MDF/colon: 9.5 ± 0.9 and 6.4 ± 0.9 in controls ($n = 8$) and SITA groups ($n = 8$), respectively; means ± SE, $p < 0.05$]. Reactive oxygen species in the blood were also significantly lower in the SITA group [6.75 ± 0.69 and 5.63 ± 0.75 (H_2O_2 in mM) in controls ($n = 5$) and SITA ($n = 6$), respectively; means ± SE, $p < 0.05$]. Rats treated with SITA had a lower DPP4 activity in the intestine but not in the plasma. Intestine growth morphometric parameters and colon proliferation, as proliferating cell nuclear antigen expression, were not affected by SITA. In conclusion, the results suggest a protective effect of DPP4i against colon carcinogenesis that could be exploited in chemoprevention trials.

Colon cancer and insulin resistance (IR), a metabolic deregulation predisposing to Type 2 diabetes mellitus (T2DM), are affected by similar diet and lifestyle factors (*e.g.,* calorie-rich food and body and abdominal fatness together with low physical activity).[1,2] Interestingly, both IR and T2DM patients are at increased risk of colon cancer.[1,2]

Inhibitors of dipeptidyl peptidase-4 (DPP4i), as well as long-acting glucagon-like peptide-1 (GLP-1) receptor agonists, have been recently used for pharmacological treatment of T2DM.[3] DPP4 is the enzyme involved in the degradation of GLP-1, the gut-derived incretin hormone stimulating insulin and suppressing glucagon secretion, and of GLP-2, which is trophic for the intestinal mucosa.[4]

The possibility that incretin-based therapies may affect colon cancer has been investigated; however, the data are fragmented, and only few studies have explored the relationship between DPP4i and colon carcinogenesis.[4–9] GLP-1 has been reported to inhibit cell growth and increase apoptosis in colon cancer cells, suggesting a protective effect against cancer.[7] However, GLP-1 analogs have also been reported to increase intestinal proliferation, a potentially dangerous effect for carcinogenesis.[5,8] As for GLP-2, it has been reported that it did not affect carcinogenesis in Min mice, a genetic model of intestinal tumorigenesis.[6] However, GLP-2 increased dysplasia and carcinogenesis in other experimental models,[4] thus suggesting an opposite effect to GLP-1. Therefore, it is possible to speculate that when the half-life of both peptides is increased by DPP4i, their overall effect on the intestine may be null. Accordingly, most reports indicate that DPP4i neither affect intestinal proliferation in rodents[5,8] nor affect colon carcinogenesis in mice, at least when high dosages are administered for a short period of time.[5] Moreover, the protein DPP4 shares high homology with the fibroblast activation protein (FAP), a protease expressed by tumor-associated fibroblasts involved in tumorigenesis, angiogenesis and metastasis.[9–11] Accordingly, DPP4i, as well as more specific inhibitors of FAP, inhibit cancer growth, suggesting a

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What's new?

The enzyme dipeptidyl peptidase-4 (DPP4) rapidly degrades insulin-stimulating incretin hormones, making its inhibition an appealing strategy for the treatment of type 2 diabetes mellitus and possibly colon cancer. The DPP4 inhibitor sitagliptin is of particular interest, though its role in colon carcinogenesis is unclear. Here, chemopreventive administration of sitagliptin in rats, at doses comparable to those used in therapeutic settings for humans with diabetes, was found to reduce precancerous colon lesions and to lower levels of reactive oxygen species in the blood. The findings warrant further investigation of sitagliptin in human chemoprevention trials for colon cancer.

protective effect of DPP4i independent from glycemic mechanisms.[9–11]

Given these considerations and the therapeutic efficacy of DPP4i in T2DM, associated with increased colon cancer risk,[2,3] we hypothesized that Sitagliptin (SITA) administered chronically at a dose similar to the therapeutic range might actually decrease colon carcinogenesis. We tested this hypothesis in 1,2-dimethylhydrazine (DMH)-induced rats, measuring the precancerous lesions mucin-depleted foci (MDF) as endpoint of colon carcinogenesis.[12] Rats were fed high-fat (HF) diet promoting IR and colon carcinogenesis.[13] In addition, as DPP4i may affect oxidative stress,[14] which contributes to the carcinogenesis process, we also tested the effect of SITA on reactive oxygen species (ROS) in the blood. Gross metabolic parameters and growth effects on the small intestine and colon were also determined.

Material and Methods

Diet composition

The HF diet composition (23% corn oil and 32% sucrose, w/w) is based on the AIN76 diet, as reported.[15] SITA (Januvia, MSD, UK) was added to the HF diet at 260 ppm (260 mg/kg of diet/day). Because rats eat ~11 g of diet/day and weight about 270 g, the estimated dosage of SITA is about 10 mg/kg body weight. On the basis of FDA preclinical studies and considering the different metabolic rate between rats and humans, this dose corresponds to the therapeutic human dose of 100 mg/day.[8,16,17]

Animals and induction of carcinogenesis

Male F344 rats, 4–5 weeks old at their arrival from the supplier (Nossan, Milan, Italy), were fed the HF diet. At 6–7 weeks of age, rats ($n = 16$) were treated with two weekly subcutaneous injections of DMH (100 mg/kg each) to induce colon carcinogenesis (experimental protocol approved by the Commission for Animal Experimentation of the Italian Ministry of Health). Six rats were treated with an equal volume of saline ($n = 6$). One week later, both DMH- and saline-treated rats were randomly allocated to two groups: (*i*) continuing with the HF diet (controls) and (*ii*) fed with the HF diet containing SITA (260 ppm) until sacrifice (SITA). In both groups, there were eight rats induced with DMH and three rats treated with saline. Animals were sacrificed 15 weeks after DMH/saline.

Collection of the samples and DPP4 activity

The intestines (small intestine and colorectum) were excised, flushed with saline, gently dried on a soft paper and weighed. A segment of jejunum and the entire colorectum were also collected and processed, as described below. ROS were determined according to Garelnabi *et al.*[18] in both whole blood and plasma. DPP4 enzymatic activity was determined in serum as well as in ileal mucosa homogenates measuring the production of *p*-nitroaniline (pNA; Sigma-Aldrich, Italy) in microwell plates with 150 μl of 1 mM solution of the DPP4 substrate Gly-Pro-pNA (Sigma-Aldrich) incubated for 60 sec at 37°C.[5,19] Linearity of the absorbance of pNA was verified with a standard curve, and the activity was expressed as nanomoles of pNA produced/μg of protein/min.

MDF determination and proliferative activity

Colorectum was longitudinally opened, fixed flat in formalin and stained with high-iron diamine Alcian Blue to highlight mucin production, as described.[12,15] MDF determination was performed blindly in the entire colorectum.[12] A segment of the distal colon of DMH-induced rats containing no lesions was then processed for proliferating cell nuclear antigen (PCNA) expression to measure proliferative activity in longitudinal crypt sections, as reported.[15]

Morphometric analysis of the intestines

Crypt depth and villus length were blindly determined in histological sections of the jejunum stained with H&E using ACT-2U software program (Nikon, Instruments Europe, Badhoevedorp, NL) connected *via* a camera to the microscope (Optiphot-2; Nikon). At least 20 well-oriented crypts and 10 villi per rat were analyzed.

Metabolic parameters

At various times during the experiment, the rats were housed individually in metabolic cages and fasted overnight. The next morning, glycemia was measured by a glucorefractometer in blood collected from the tail vein. A satiety test was also performed transferring overnight-fasted rats into a cage with a weighed amount of food (standard laboratory pellets). The amount consumed was measured at 30, 60, 90 and 120 min after addition of the pellets to the cage. Cumulative values at 120 min are given.

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Statistical analysis

Comparisons among the four different groups [also including the saline-treated rats (both SITA and controls)] were performed with two-way ANOVA, after verification of the homogeneity of variance, with Cochran's and Bartlett's tests. We analyzed at the same time the effect of SITA (in saline- and DMH-induced rats) and DMH (in controls and SITA-treated rats), as well as the possible interactions between the two treatments. When the comparison was between two groups (DMH-induced rats treated with SITA compared to DMH rats not treated), a *t*-test was performed. Level was fixed at 0.05, two sided, in both cases. Statistical calculations were performed using GraphPad Instat (GraphPad Software, CA) and Statgraphics Statistical Packages (Statistical Graphic Corporation, Rockville, MD).

Results

Body weight and metabolic parameters

Body weight was not affected by SITA treatment (Table 1); similarly, fasting glycemia was similar between controls and SITA-treated rats; however, DMH-induced rats had a significantly higher glycemia than saline-treated counterparts (Table 1). The amount of food eaten in 2 hr after overnight fasting (satiety) was: 2.2 g ± 0.7 (4), 1.8 g ± 0.3 (3), 2.8 g ± 0.3 (6) and 3.5 g ± 0.5 (6) in saline-treated (controls and SITA) and in DMH-induced rats (controls and SITA), respectively [means ± SE (determinations per group)]. The statistical analysis showed that the effect of SITA on food consumption was null, whereas the effect of DHM did not reach statistical significance, although a trend toward an increase ($p = 0.059$) was observed.

ROS in the blood

ROS determined at sacrifice in the blood of DMH-induced rats were significantly lower ($p < 0.05$) in SITA-treated rats than in controls (Fig. 1*a*). A similar result, although not statistically significant, was observed measuring ROS in the frozen plasma of saline-treated rats [H_2O_2 in mM: 9.7 ± 1.5 and 7.73 ± 0.6 in controls ($n = 3$) and SITA-treated rats ($n = 3$), respectively (mean ± SE)].

Colon carcinogenesis

Rats treated with SITA had a significantly lower number of the precancerous lesions MDF in the colorectum than controls (Fig. 1*b*), indicating a protective effect of SITA against carcinogenesis. The number of crypts forming each focus was not affected by SITA (data not shown).

Morphometric measurements of the intestines and DPP4 activity

The weight of the intestines, normalized on body weight, was similar across groups (Figs. 2*a* and 2*b*). Colon proliferation, determined as PCNA expression in DMH-induced rats, was unchanged (Fig. 2*c*). The number of cells/colonic crypts was also similar across groups (data not shown). Morphometric measurements of the small intestine (Figs. 2*d* and 2*e*) also did not show any effect of SITA or DMH treatment. DPP4 activity measured in ileal mucosa homogenates was significantly lower in rats treated with SITA than in controls (Fig. 2*f*). On the contrary, DPP4 activity in the serum was not significantly modified by SITA (data not shown).

Discussion

The main finding of our study is that SITA, a DPP4i with clinical effectiveness in the treatment of human T2DM, decreases colon carcinogenesis and blood ROS levels when administered to rats at a dosage similar to the human therapeutic setting.

The results were obtained measuring the precancerous lesions MDF in DMH-induced rats, a robust experimental model to identify drugs or dietary treatments with chemopreventive activity against colon cancer.[1,5,12,13] Rats were fed a

Table 1. Rat body weight and fasting glycemia during the various phases of the experiment

Groups		Start of the experiment, before DMH/saline	After DMH/saline before SITA	After SITA
Body weight (g)				
DMH	SITA (8)	124.0 ± 4.1 (16)	164.9 ± 5.7 (8)	358 ± 10 (8)
	Controls (8)		154.6 ± 4.9 (8)	352 ± 10 (8)
Saline	SITA (3)	123.7 ± 3.4 (6)	168.0 ± 4.4 (8)	380 ± 12 (8)
	Controls (3)		178.3 ± 5.8 (8)	368 ± 15 (8)
Glycemia (mg/dl)				
DMH	SITA (8)	86.3 ± 0.5 (3)	95 ± 3.3 (4)	99.8 ± 5.6 (11)[1]
	Controls (8)		82 ± 6 (4)	101.1 ± 4.6 (10)[1]
Saline	SITA (3)		ND	87.3 ± 2.3 (3)
	Controls (3)		ND	83.7 ± 7.6 (4)

Numbers in parentheses represent the number of animals/determinations per group.
[1]Significantly different from saline-treated rats at the same time after SITA ($p = 0.046$ with two-way ANOVA). Abbreviation: Not determined.

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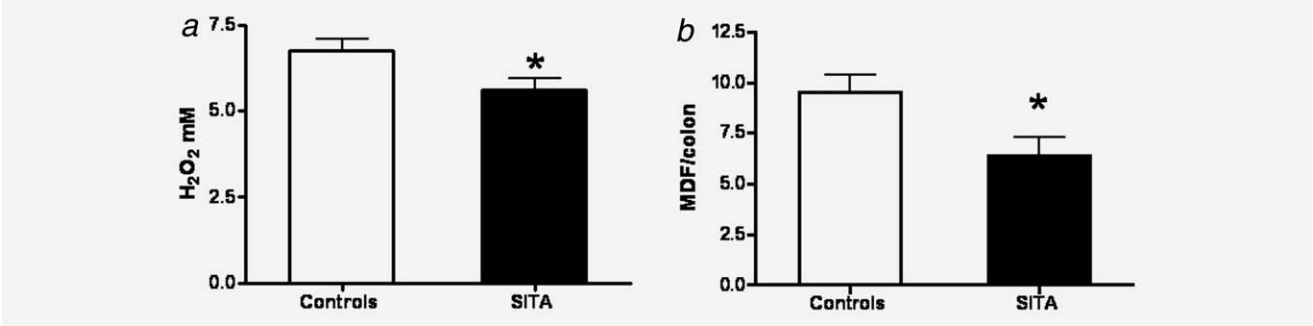

Figure 1. (*a*) Blood ROS in DMH-induced rats treated with Sitagliptin (SITA) or not (controls). Values are mean ± SE, $n = 6$; *$p = 0.045$ comparing SITA with controls. (*b*) MDF/colon in DMH-induced rats treated with SITA or not (controls). Values are mean ± SE, $n = 8$; *$p = 0.02$ comparing SITA with controls (*t*-test).

HF/high-sucrose diet, mimicking human diets leading to both increased colon carcinogenesis and IR.[13]

To the best of our knowledge, this is the first study reporting a protective effect of the DPP4i SITA on intestinal carcinogenesis.

Recently, Kissow *et al.*[5] reported that SITA administered to mice at a dose of 400 mg/kg (about 40 times higher than the dose we used) did not affect DMH-induced carcinogenesis. At variance with our long-term SITA administration (13–14 weeks), in the report of Kissow *et al.,* the drug was administered for a much shorter period (6–7 weeks), a factor that could have hampered the potential positive effects of SITA. Moreover, in the study of Kissow *et al.,* carcinogenesis was induced with several repeated injections of DMH (12 weekly treatments), administering SITA only after a long period (16 weeks) from the last injection with DMH.[5] On the contrary, we administered SITA starting from the very early steps of carcinogenesis, that is, few days after the second (and last) treatment with DMH, and thus allowing SITA to counteract the process of carcinogenesis from the very beginning. Interestingly, the effect of SITA on carcinogenesis occurs independently from variation of proliferation and morphometric parameters of the colon and small intestine. This result is in agreement with previous reports documenting that NN-7201, a DPP4i administered at a dosage similar to that we used, did not affect intestinal growth in rats with normal colon.[8] However, it has also been reported that SITA enhanced intestinal adaptation in mice with bowel resection, an effect that could be explained with the pathological condition of those mice and with the high dosage of SITA causing

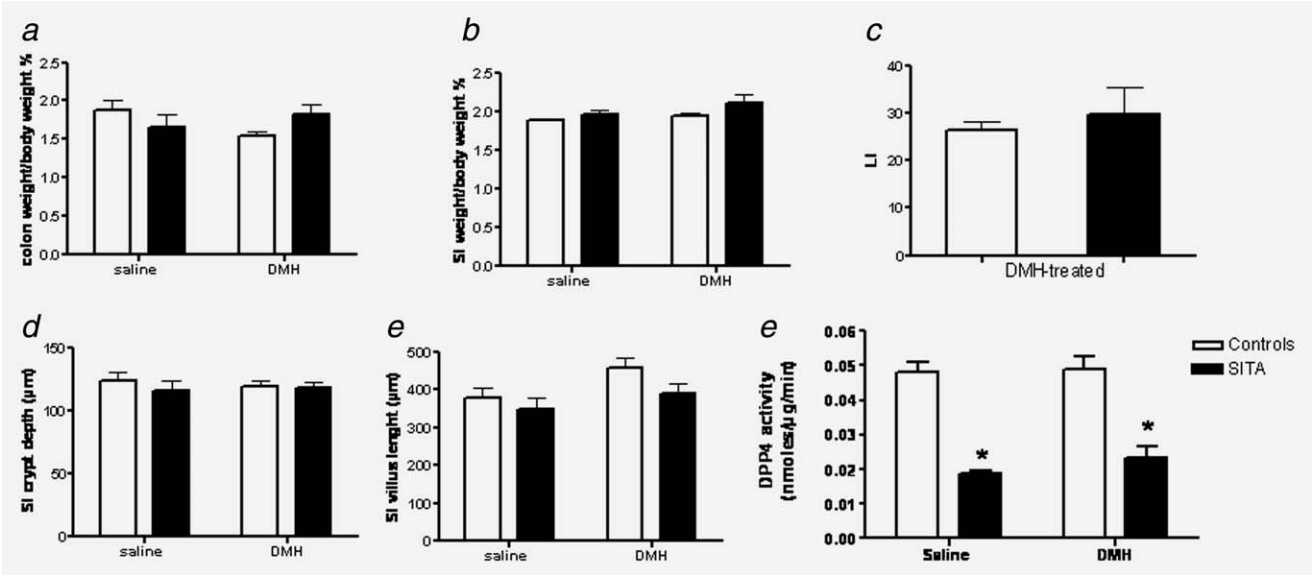

Figure 2. Colon weight/body weight % (*a*) and small intestinal weight/body weight % (*b*) in the different groups. (*c*) Expression of PCNA as labeling index (LI: number of labeled cells/cells counted × 100) in the colon mucosa of DMH-induced rats treated or not with SITA; bars are mean ± SE, $n = 7$ and 8 in controls and SITA-treated rats, respectively. (*d* and *e*) Depth of crypts and length of the villi in the small intestine, respectively, in the different groups; bars are mean ± SE, $n = 3$ and 8 in each group of the saline and DMH-treated rats, respectively. (*f*) DPP4 activity (nanomoles of pNA produced/µg of protein/min) in the ileal mucosa; $n = 2$, 3, 6 and 5 in saline (controls and SITA) and DMH-treated (controls and SITA) rats, respectively. *$p = 0.01$ comparing SITA with controls in both saline and DMH-treated rats (two-way ANOVA). In each graph, white bars represent controls, and the black bars represent SITA-treated rats.

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increased level of GLP-2 (actually, GLP-1 was not tested in that study).[20] It is also possible that in our experimental conditions, SITA exerts specific inhibitory effects on preneoplastic cells. Accordingly, recent data showed that GLP-1 has protective effects on colon cancer cell lines and in transplanted tumors[7]; thus, although we did not test the level of GLP-1 in our rats, it would be possible to speculate that the beneficial effect of SITA that we found is partially related to an increase in GLP-1. Although we did not find changes of DPP4 enzymatic activity in the serum, the enzymatic activity in the ileum was significantly lower in the rats receiving SITA, thus suggesting a local effect of this drug. Accordingly, a recent study in mice treated with various doses of SITA reported that relatively low doses of this drug, as in our study, inhibited DPP4 activity in the intestine but not in the plasma.[19]

On the other hand, it is also interesting to consider that DPP4 shares high homology with the FAP expressed by tumor-associated fibroblasts and related to increased tumorigenesis.[9,10] Overexpression of FAP has been reported in colon carcinogenesis,[10] and inhibitors of FAP have been evaluated in Phase II trials for metastatic colon cancer.[11] It has also been reported that vildagliptin, a DPP4i related to SITA, as well as synthetic inhibitors of FAP inhibit lung tumorigenesis,[9] suggesting a protective effect of DPP4i independent from glycemic mechanisms.

In fact, in our study, the effect of SITA on carcinogenesis occurred without any associated metabolic action on either body weight or blood glucose. Incidentally, we observed that the DMH-treated rats (irrespectively from the SITA treatment) had higher glycemia than their saline counterpart. A similar effect of the carcinogen DMH on glucose blood levels has been reported in the past; however, besides a generic toxic effect of DMH on glucose metabolism, no clear explanation exists for this phenomenon, which certainly deserves further investigation.[21]

We also found that rats treated with SITA had blood ROS lower than controls, suggesting that SITA might also work as a scavenging or an antioxidant compound, helping to limit ROS-mediated tissue damages. In agreement with our result, Vaghasiya *et al.*[14] demonstrated that SITA protects against ROS-mediated renal damage induced by hypoxia in rats. Similar protective effects against ROS were reported for GLP-1 in reducing cardiac ischemia reperfusion injuries.[22]

Our study has some limitations. In fact, the results have been obtained with a single dosage of SITA administered for only one period of time. However, the results are clear-cut, and the level of MDF inhibition is comparable to that we observed with known colon cancer chemopreventive regimens (*e.g.,* low-fat diet or caloric restriction).[23,24] Therefore, even with the due caution in extrapolating results from animals to humans, these data may be particularly relevant in T2DM patients considered at risk for colon cancer. In addition, provided further experimental studies to understand the mechanism of action of SITA on colon carcinogenesis, its chemopreventive activity might also justify intervention trials in nondiabetic patients at risk for colon cancer such as patients operated for polyps or colon cancer. In fact, the almost absent risk of hypoglycemia allows the indication of DPP4i to nondiabetic patients as well. Besides, although the duration of available clinical trials with DPP4i is too short to provide any reliable information on the effect of these drugs on the incidence of cancer in humans, no reasons for concern have emerged so far.[25]

In conclusion, the results of our study show that SITA, a DPP4i, given chronically to DMH-induced rats reduces precancerous lesions in the colon and blood ROS, suggesting a protective effect toward the carcinogenesis processes, which deserves further investigation.

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