Metformin prevents hormonal and metabolic disturbances and 1,2-dimethylhydrazine-induced colon carcinogenesis in non-diabetic rats

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ABSTRACT
Effects of two doses of the anti-diabetic drug, metformin (MF), on hormonal and metabolic levels of serum of non-diabetic male Wistar rats with 1,2-dimethylhydrazine (DMH)-induced colon tumor adenocarcinomas were studied. Carcinogenesis in the animals was also observed. Rats with DMH-induced colon adenocarcinomas had elevated levels of serum glucose, insulin, insulin-like growth factor-1, total cholesterol, triglycerides, catalase, malonic dialdehyde, glycated hemoglobin, aspartate aminotransferase, and alanine aminotransferase and decreased hemoglobin. Treatment with two doses of MF normalized majority of these changes in DMH-treated rats, whereas the drug was ineffective in rats without DMH treatment. The only exception was the decreased triglyceride levels in MF-treated rats. A 100 mg/kg dose of MF increased DMH-induced exophytic colon carcinomas and decreased endophytic tumors compared with untreated rats. Moreover, both MF doses increased DMH-induced and highly differentiated tumors and decreased the invasiveness of colon carcinomas compared with rats provided with DMH and water. Therefore, effects of MF on metabolic homeostasis are critical for preventing colon cancer.

KEYWORDS
Colon cancer; prevention; 1,2-dimethylhydrazine; metformin; rat

Introduction
Colorectal cancer (CRC) is the third most common malignancy in humans worldwide; more than one million new cases occur annually1. Age is a leading CRC risk factor. More than 90% of CRC cases occur in people aged over 50, and approximately 75% of the cases are diagnosed in people older than 65. Furthermore, risks starting at 40 years of age increase sharply at 50 and double each decade until age 80. Hyperinsulinemia and obesity are key factors in cancer pathogenesis, including CRC3-6. The chemical carcinogen, 1, 2-dimethylhydrazine (DMH), has been widely used for induction of colon cancer. Regardless of mode of administration, DMH specifically induces tumors within the descending colon of rats and some mouse strains, and resulting histopathologies are similar to those observed in human sporadic colon tumors7,8. Meanwhile, the anti-diabetic biguanide, metformin (MF), lowers elevated insulin levels in type 2 diabetes3,9, significantly reduces various cancer risks in humans with the condition, and prevents tumor development in numerous rodent tissues and organs10,11. Furthermore, MF inhibits some chemically induced colon carcinogenesis in diabetic, obese12, and non-diabetic rats13. In this work, we showed the inhibitory effects of MF on DMH-induced colon carcinogenesis in non-diabetic rats. Such effects mainly involve the normalizing influence of MF on hormonal and metabolic homeostases.
seven rats were kept in T3-type cages under a standard light/dark regimen (12 h light: 12 h darkness) at (22±2)°C and received standard laboratory PK-120 (Laboratorkorm, Russia)\textsuperscript{14} and tap water ad libitum. Animals were checked daily by animal care personnel and weekly by a veterinarian. The weights were measured weekly as well. The study was conducted per the regulations for ensuring humane treatment of animals under the approval of the Committee on Animal Research of N.N. Petrov Research Institute of Oncology.

**Chemicals**

DMH was provided by Sigma Chemical Co., St. Lois, MO, USA and kept at –20°C. MF (MF HCl, Siophor) was purchased from Berlin-Chemie, Menarini Group, Germany.

**Experiment 1**

A total of 58 male Wistar rats with 2-month-old were randomly subdivided into 6 groups. A total of 24 rats in groups 1–3 were not exposed to carcinogens, whereas 34 rats from groups 4–6 were administered with 5 subcutaneous DMH injections weekly at a single dose of 21 mg/kg of body weight (calculated as a base). In this regimen, carcinogens induced colon tumors in majority of rats\textsuperscript{7}. DMH was ex tempore dissolved in normal saline and neutralized with sodium bicarbonate (pH 7.0). Starting from the first carcinogen injection, groups 1 and 4 were provided with 1 mL tap water via intragastric gavage, whereas groups 2 and 5 were administered daily with MF (100 mg/kg) via gavage. Groups 3 and 6 were given MF (300 mg/kg) dissolved in 1 mL tap water. This treatment was concluded 2 months after the first DMH injection. The experiment was finalized six months after the first carcinogen injection. After being sacrificed by ether vapor, rats were autopsied by longitudinally opening the intestines. Tumor position and size were recorded\textsuperscript{7}. After histological processing, tissues were embedded in paraffin. Histological sections measuring 3 μm thick were stained with hematoxylin-eosin and microscopically examined; in the experimental group, examination was performed as blind process. Tumors were classified per International Agency for Research on Cancer recommendations\textsuperscript{15}.

**Statistical analysis**

Experimental results were statistically processed following variation statistics using Statistica-10. All data were expressed as mean ± standard error (Figures 1 and 2) or confidence interval for the standard deviation (Table 1). The significance of discrepancies was defined according to Chi-square analysis between experimental and control groups (Table 1). Differences in estimated parameters among the groups were assessed using non-parametric criterion of Mann-Whitney U test (Figures 1 and 2)\textsuperscript{16}. P<0.01 and 0.05 were considered as significant.

**Results**

**Effect of MF on DMF-induced hormonal and metabolic disturbances in male rats**

MF treatment failed to influence weight gain in both non- (groups 2 and 3) and DMH-exposed rats (groups 5 and 6). Thus, MF did not significantly affect weight gain between non- and DMH-exposed groups (data not shown). Two-month administration of both doses of MF to non-exposed rats significantly decreased triglyceride serum levels and failed to influence other metabolic parameters (Figure 1). Dramatic parameter disturbances were observed in DMH and water-treated rats. The animals were sacrificed six months after the first carcinogen injection. Compared with
Figure 1 Effect of 1,2-dimethylhydrazine (DMH) and metformin on hormonal and metabolic parameters in the serum male Wistar rats. (A) glucose. (B) Insulin. (C) IGF-1. (D) Total cholesterol. (E) Triglycerides. (F) Cu, Zn-superoxide dismutase. (G) Catalase. (H) Malonic dialdehyde. (I) Hemoglobin. (J) Glycated hemoglobin. (K) Alaninaminetransferase. (L) Aspartataminetransferase. (M) VEGF. Data presented as mean±SEM, \( n=6–15 \) per group. \( P<0.05 \). a: DMH vs. control; b: DMH+MF vs. DMH. Rats bearing DMH-induced colon adenocarcinomas have elevated serum level of glucose, insulin, IGF-1, total cholesterol, triglycerides, catalase, malonic dialdehyde, glycated hemoglobin, AST, ALT and decreased level of hemoglobin. Treatment with MF in both doses normalized majority of these changes in DMH-treated group of rats, whereas failed to modify them in rats not treated with DMH. Only exception was decreased level of triglycerides in MF-treated rats (Figure 1E, \( P<0.05 \)).
the control group, non-treated rats had increased levels of glucose (+25.6%), insulin (+36.2%), IGF-1 (+37.1%), total cholesterol (+47.4%), and triglycerides (+106.9%) and increased activities of catalase (+35.3%), MDA (+33.3%), AST (+93.8%), ALT (+71.4%), VEGF (+65.5%), and glycated hemoglobin (+56.7%). SOD activity did not change significantly (+19.2%, *P >0.05). Both MF doses alleviated carcinogenic effects. Majority of parameters were normal, and indices covered those DMH-unexposed and MF-untreated rats (Figure 1A–1M).

### Effects of MF on DMH-induced colon carcinogenesis in male rats

In Experiment 2, intestinal tumors were found in majority of DMH-exposed rats (Table 1).

In group 1 (DMH+water), all rats developed colon tumors (100%). Tumor incidences in different colon parts in group 1 varied: 63% in ascending colon, 100% in descending colon, and 25% in rectum. Moreover, 76.7% of colon tumors were observed in descending colons, 16.7% in ascending colons, and 6.7% in rectums. Maximal effect of 100 mg/kg daily MF dose was observed in the ascending colon, in which DMH-induced carcinogenesis was also completely inhibited (Table 1). The two MF doses did not affect colon carcinoma incidence in rat rectums and descending colons. Higher MF dose (300 mg/kg) was less effective in suppressing colon carcinogenesis compared with lower amounts (100 mg/kg).

Macroscopically, neoplasms are exophytic or endophytic. Microscopically, malignant intestinal tumors have different types, among which tubular adenocarcinomas are predominant. All carcinoma types are typical in DMH-induced neoplasms. Table 1 and Figure 2 present the data on the effects of MF on DMH-induced colon tumor development.

Morphological analysis showed that tumors with exophytic growth patterns developed more frequently in the group treated with 100 mg/kg MF compared with DMH + water.

### Table 1 Colon tumors localization, incidence, multiplicity and size in rats exposed to 1,2-dimethylhydrazine (DMH) and metformin

| Parameters                           | DMH + water | DMH + metformin, 100 mg/kg | DMH+metformin, 300 mg/kg |
|--------------------------------------|-------------|----------------------------|--------------------------|
| No. of rats                          | 8           | 9                          | 7                        |
| Ascending colon                      |             |                            |                          |
| No. of tumor-bearing rats            | 5 (63%)     | 0                          | 2 (29%)                  |
| No. of tumors                        | 5           | 0                          | 5                        |
| No. of tumors per tumor-bearing rat  | 1.0         | 0                          | 2.5                      |
| Mean size of tumors, mm$^2$          | 47±37.8     | 0                          | 13±8.5*                  |
| Descending colon                     |             |                            |                          |
| No. of tumor-bearing rats            | 8 (100%)    | 6 (67%)                    | 6 (86%)                  |
| No. of tumors                        | 23          | 12                         | 19**                     |
| No. of tumors per tumor-bearing rat  | 2.9         | 2                          | 3.2                      |
| Mean size of tumors, mm$^2$          | 93±79.5     | 37±31.2*                   | 43±23.9*                 |
| Rectum                               |             |                            |                          |
| No. of tumor-bearing rats            | 2 (25%)     | 2 (22%)                    | 2 (29%)                  |
| No. of tumors                        | 2           | 2                          | 2                        |
| No. of tumors per tumor-bearing rat  | 1           | 1                          | 1                        |
| Mean size of tumors, mm$^2$          | 26±24.9     | 49±37.3                    | 146±66.8                 |
| Total colon                          |             |                            |                          |
| No. of tumor-bearing rats            | 8 (100%)    | 7 (78%)                    | 6 (86%)                  |
| No. of tumors                        | 30          | 14                         | 26                       |
| No. of tumors per tumor-bearing rat  | 3.75        | 2.0                        | 4.8                      |
| Mean size of tumors, mm$^2$          | 81±72.9     | 39±27.5*                   | 45±22.7*                 |

The difference in the parameter for rats exposed to DMH+water is significant, *P<0.01; The difference in the parameter for the group DMH+metformin–100 is significant as well: **P<0.01.
group. Opposite results were observed with endophytic colon
tumors (Figure 2A). The group that was treated with lower
MF dose had less invasive (Figure 2B) and more
differentiated tumors (Figure 2C) compared with the DMH + water group. Figure 3 shows microphotographs of the
observed colon adenocarcinoma types. Tumor size
distribution analysis showed that in descending colons of
DMH + water and DMH + MF groups, 300 small tumors (<
10 mm²) appeared less frequently compared with the MF
group with 100 mg/kg dose (26%, 26%, and 50%,
correspondingly). Thus, these data indicate the inhibitory
effects of MF on DMH-induced colon carcinogenesis.

**Discussion**

The DMH-induced carcinogenesis in epithelial cells includes
the following: formation of most active metabolites
(methylazoxymethanol or methyldiazohydrate) in the liver;
binding of metabolites to glucuronic acid; delivery of
conjugates to intestines via blood flow; release of active
metabolites through enzymatic activity of intestinal flora (ß-
glucuronidase); formation of carbonium ion (CH₃⁺); specific
methylation of macromolecules, which in enterocytes, are
mainly DNA at O⁶ position of guanine; miscoding effects⁷.
These events result in mutation and activation of Ki-ras
oncogene and inactivation of p53¹⁷. Moreover, reports
presented the significant role of free radicals in DHM-
induced colon carcinogenesis¹⁸,¹⁹. The present study
confirmed the effects of DMH on oxidative stress parameters.
Furthermore, we showed the normalizing effect of MF on
MDA levels in DMH-treated rats (Figure 1). Bordini et al.²⁰
observed similar effects of MF in azoxymethane (AOM)-
exposed mice. Shortly after starting DMH treatment, exposed
organisms experienced significant disturbances in their
neuroendocrine and immune systems and lipid and
carbohydrate metabolisms. DMH treatment was followed by
an increase in sensitivity threshold of the hypothalamus to
inhibition by estrogen²¹, decrease in hypothalamic biogenic
amine content²², and disturbances in diurnal rhythms at
biogenic amine levels in hypothalamic nuclei of rats²³. Anti-
diabetic biguanide treatment alleviated immunodepression in
rodents exposed to DMH²⁴ and AOM²⁵. We observed
increased levels of glucose, insulin, IGF-1, total cholesterol,
triglycerides, MDA, glycated hemoglobin, and VEGF in
serum of rats with DMH-induced colon tumors compared
with the group without DMH (Figure 1). These findings

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**Figure 2** Effect of metformin on some parameters of 1,2-dimethylhydrazine-induced colon carcinogenesis in male Wistar rats. Data presented as mean±SEM, n=7–9 per group. * P<0.01; § P<0.05 vs. DMH group. Treatment with MF in dose 100 mg/kg increased relative number of induced with DMH exophytic colon carcinomas and decreased number of endophytic tumors. Both doses of MF increased relative number of DMH-induced highly differentiated tumors and decreased invasiveness of colon carcinomas as compare with group given DMH with water.

**Figure 3** Microphotographs of 1,2-dimethylhydrazine-induced colon adenocarcinomas. (A) Highly differentiated adenocarcinoma. (B) Moderately differentiated adenocarcinoma. (C) Low differentiated adenocarcinoma (H&E staining, 70×).
agree with available data\textsuperscript{12,13,26,27}. Furthermore, we observed the normalizing effects of both MF doses on these parameters in DMH-exposed rats (Figure 1). Notably, AST and ALT levels were not different in rats treated with higher and lower MF doses, thereby suggesting the non-toxicity of MF on liver functions.

Table 2 summarizes the data on inhibitory effects of anti-diabetic biguanides on colon carcinogenesis. In most studies, anti-diabetic biguanides inhibited AOM- or DMH-induced colon carcinogenesis in mice and rats. Detailed analysis of experimental results are provided elsewhere\textsuperscript{28,29}. MF treatment was followed by decreased levels of proliferation indices, which were evaluated with 5-bromodesoxyuridine, proliferating cell nuclear antigen indices, phosphorylated mechanistic target of rapamycin (mTOR), S6 kinase, and S6 proteins, as revealed by Western blot analysis, in the colonic mucosa of AOM-treated mice\textsuperscript{30}. The authors believe that MF suppresses colonic epithelial proliferation by inhibiting the mTOR pathway through S' adenosine monophosphate-activated protein kinase activation. However, MF did not affect the level of O\textsuperscript{6}-Methylguanin in the colon or liver of AOM-treated mice. Results showed that MF did not affect the AOM alkylation capacity and carcinogenicity. Therefore, the normalizing effect of MF on neuroendocrine and hormonal metabolic shifts, which occurred in rodents during colon carcinogenesis, are critical in colon cancer prevention.

Zaafar et al.\textsuperscript{32} studied the effects of MF on cancer development in diabetic and non-diabetic mice. In diabetic mice, MF treatment alone increased the number of surviving mice compared with the diabetic DMH group. Moreover, MF significantly reduced histopathological scores in diabetic mice colon. Serum VEGF levels in non-diabetic DMH group were not significantly higher than those of non-diabetic saline groups. In non-diabetic mice, MF reduced the serum concentration of VEGF compared with those in the non-diabetic/DMH control. In our study, we observed a tendency toward increased VEGF level in DMH-treated rats compared with control animals, which were not exposed to carcinogens, whereas MF did not influence this parameter. Statistical analysis showed a significantly reduced histopathologic score for colon mucosa of MF-treated diabetic mice compared with DMH control, whereas in non-diabetic animals, the drug failed to improve the scores. This study highlighted the high susceptibility of diabetic rodents to carcinogenic effects of DMH. Inhibitory effects of MF on DMH-induced colon carcinogenesis were observed in Sprague Dawley rats with type 2 diabetes; the condition was induced with small dose of streptozotocin combined with a high-fat diet\textsuperscript{32}. MF treatment was followed by decreases in ACF number, colonic tissue proliferation, and colon tumor incidence, multiplicity, and size. These results and other studies confirmed the role of diabetes as risk factor for cancer and established a connection between glucose levels and development of micro- and macro-vascular complications\textsuperscript{37,38}. Our data agree with the other studies, showing that MF is effective in cancer prevention in both diabetic and non-diabetic animals that are exposed to DMH or AOM (Table 2)\textsuperscript{10,11}. Moreover, clinical trials demonstrated the decrease in colon cancer risk of type 2 diabetes and non-diabetic patients\textsuperscript{39-51}. Meta-analysis of 37 studies

| Species, strain | Sex          | Carcinogenic agent | Drug | Doses          | Route   | Effect  | Reference |
|-----------------|--------------|--------------------|------|----------------|---------|---------|-----------|
| BALB/c mice     | Male & Female| AOM                | MF   | 250 mg/kg     | Diet    | Inhibition | 30        |
| BALB/c mice     | Male & Female| AOM                | MF   | 250 mg/kg     | d.w.    | Inhibition | 31        |
|                  |              |                    | MF   | 250 mg/kg     | i.p.    | Inhibition | 31        |
| BALB/c mice     | Male & Female| AOM                | MF   | 250 mg/kg     | i.p.    | Inhibition | 25        |
| BALB/c mice     | Female       | DMH                | MF   | 250 mg/kg     | i.p.    | Inhibition | 20        |
| Swiss albino mice| Male        | DMH                | MF   | 100–200 mg/kg | Oral    | Inhibition | 32        |
|                |              |                    | MF   | 240 mg/kg     | Oral    | Inhibition | 13        |
| F344 rats       | Male         | AOM                | MF   | 15 mg/kg      | d.w.    | Inhibition | 27        |
| F344 rats       | ND           | AOM                | MF   | 500–1000 ppm  | Diet    | No effect  | 33        |
| F344 rats       | ND           | AOM                | MF   | 1000 ppm      | Diet    | No effect  | 34        |
| LIO rats        | Female       | DMH                | PF   | 5 mg/rat      | Oral    | Inhibition | 35        |
| LIO rats        | Female       | DMH                | MF   | 0.1 mg/ml     | d.w.    | Inhibition | 36        |
| SD rats         | Male         | DMH                | MF   | 150 mg/kg     | Oral    | Inhibition | 12        |
| Wistar rats     | Male         | DMH                | MF   | 40–360 mg/kg  | Oral    | Inhibition | 13        |

AOM: azoxymethane; DMH: 1,2-dimethylhydrazine; DSS: dextran sodium sulfate. d.w.: drinking water; i.p.: intraperitoneally; ppm: parts per million.
comprising more than 1.5 million participants showed that risk of colon cancer mortality was reduced by 23% in MF users compared with non-users. Thus, most epidemiological data and clinical trial results present sufficient evidence of MF efficacy in CRC prevention and treatment in humans. Furthermore, low daily MF dose (500 mg) was effective in reducing cancer risk in type 2 diabetes patients. Our findings on efficacy of lower MF dose in rats agree with clinical data. Given the practical significance of these observations, future investigations are needed to elucidate the advantage of using lower doses of the drug.

Acknowledgments

This work was supported in part by a grant from the Russian Foundation for Basic Research (Grant No. 14-04-01653). Authors are very thankful to Dr. A.V. Panchenko and Dr. M.A. Zabezhinski for help and valuable advice during the study.

Conflict of interest statement

No potential conflicts of interest are disclosed.

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