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IMPACT OF DIHYDROPYRROL DERIVATIVE ON THE NORMAL COLONIC MUCOSA OF DMH-INDUCED COLON CANCER RATS COMPARED WITH 5-FLUOROURACIL

H.M. Kuznietsova, O.V. Ogloblya, V.K. Rybalchenko*
Taras Shevchenko National University, Kyiv 01601, Ukraine

***Aim*: To compare the effects of cytostatic compound dihydropyrrol derivative (D1, 5-amyno-4-(1,3-benzothyazol-2-yl)-1-(3-methoxyphenyl)-1,2-dihydro-3H-pyrrol-3-one) and 5-fluorouracil (5-FU) on the normal colonic mucosa of tumor-bearing rats and to estimate the relationships between proliferation of normal colonic mucosa and tumor growth parameters. *Methods*: 1,2-dimethylhydrazine (DMH) carcinogenic model was used. Male Wistar rats were treated by dimethylhydrazine (20 mg/kg of body weight (b.w.) weekly) for 20 weeks, by D1 (2.3 mg/kg of b.w. daily) for 7 or 27 weeks, and by 5-FU (45 mg/kg of b.w. weekly) for 7 weeks. The number of tumor and tumor total area in dissected colon, mitotic and crypt fission indices in surrounding colon mucosa were measured and correlations between these parameters were computed. *Results*: The number of tumor node and tumor total area under the influence of D1 and 5-FU were decreased by 40–54%. D1 administration has resulted in the more gentle effect on surrounding healthy colon mucosa comparing to 5-FU, particularly, on vascular bed and proliferative activity. The changes in colon mucosa proliferative activity correlate with tumor growth parameters depending on the action of D1 or 5-FU. *Conclusions*: D1 manifests the same antitumor activity but less toxicity comparing to 5-FU that allow to suggest its possible use as an anticancer mean. Obtained correlations could be useful for better understanding of the processes preceeding the malignant transformation and their pharmaceutical correction.**
***Key Words*: 1,2-dimethylhydrazine-induced carcinogenesis, colon mucosa, dihydropyrrol derivative, 5-fluorouracil.**

Cancer is a major public health problem in all countries [1]. Statistical data have shown that malignant neoplasia remain a second cause of disease-related deaths after cardiovascular diseases [2], but demonstrate the tendency to overtake it. In Ukraine, about 160,000 new cases of cancer are diagnosed annually [3]. Unfortunately, conventional cytotoxic chemotherapy has high frequency and severity of adverse effects on the normal, actively proliferating tissues. This is the one of the main factors for choosing the proper chemotherapy and for outcomes of the therapeutical treatment [4, 5]. Therefore, the development of effective and selective anticancer drugs is a important and actual task. Targeted therapeutic agents, e.g., protein kinase inhibitors, have a high specificity to malignant cells and low toxicity [6–9], but their influence on the organism had not been thoroughly studied. In particular, the information about histopathology of healthy tissues of the cancer patients is extremely scarce. So, the design, extensive *in vivo* testing and profound analysis of adverse effects of the novel targeted protein kinase inhibitors are warranted.

Dihydropyrrol derivates, synthesized as a novel tyrosine kinase ATP-binding site blockers [10], show *in vitro* cytostatic activity against the cell lines, such as HT29, HCT-15, and COLO-205 (colorectal cancer) [11] that suggests their potential anticancer activity. The current investigation was aimed to study the effects of dihydropyrrol derivative D1 on tumor growth and proliferative activity of surrounding mucosa in colorectal cancer bearing rats. For comparison, the effects of 5-fluorouracil (5-FU), a conventional therapeutic agent for colon cancer treatment [5, 12], was concomitantly investigated.

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Correspondence: E-mail: gala_kuznetsova@rambler.ru
Abbreviations used: D1 – dihydropyrrol derivative 5-amyno-4-(1,3-benzothyazol-2-yl)-1-(3-methoxyphenyl)-1,2-dihydro-3H-pyrrol-3-one; 5-FU – 5-fluorouracil; DMH – 1,2-dimethylhydrazine.

MATERIALS AND METHODS

Animals. Sixty male Wistar rats weighing 120–130 g were studied. Animals were kept in the standard vivarium conditions. All experimental procedures executed with animals were in compliance with the European Community Council Directive.

Chemicals. D1 (5-amyno-4-(1,3-benzothyazol-2-yl)-1-(3-methoxyphenyl)-1,2-dihydro-3H-pyrrol-3-one) (Fig. 1) was synthesized as a protein tyrosine kinase inhibitor at the Department of Chemistry of the Taras Shevchenko National University of Kyiv [10].

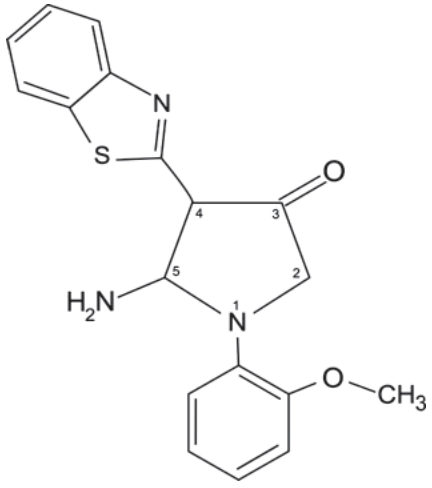

Fig. 1. Dihydropyrrol derivative 5-amyno-4-(1,3-benzothyazol-2-yl)-1-(3-methoxyphenyl)-1,2-dihydro-3H-pyrrol-3-one

D1 has *in vitro* antiproliferative activity and low *in vivo* intestine toxicity [10, 13]. The agent was dissolved in vegetable oil containing 15% dimethylsulfoxide. Animals were treated by D1 at a dose of ≈ 2.3 mg/kg of body weight (b.w.) *per os* daily for 7 or 27 weeks. Saline solution of 5-FU ("Darnitsa", Ukraine) was intraperitoneally

injected into animals at a dose of 45 mg/kg b.w. weekly for 7 weeks, as previously described [14]. 1,2-dimethylhydrazine (DMH) (Acros Organics, USA), a highly specific colorectal carcinogen in rodents, was dissolved immediately before use in saline solution adjusted to pH 6.5 with sodium hydroxide. To induce tumor development, animals were subcutaneously injected with 20 mg/kg b.w. DMH weekly for 20 weeks [15].

Experimental design. The rats were divided into 6 groups: vehicle-treated control (group 1), DMH-treated (group 2), DMH+D1 27-week-treated (group 3), DMH+D1 7-week-treated (group 4), DMH+5-FU-treated (group 5), DMH+D1+5-FU-treated (group 6) (Fig. 2). In groups 2–6, the rats were injected with 20 mg/kg b.w. DMH weekly for 20 weeks, whereas, in the control group, the rats were similarly injected with the same volume of vehicle (0.1 ml). In group 3, the rats also daily ingested 2.3 mg/kg b.w. D1 for 27 weeks, whereas in groups 1–2, the rats daily ingested the same volume of vehicle (0.1 ml) for 27 weeks. In groups 4–6, the weekly injections of 20 mg/kg b.w. DMH and the daily ingestions of 0.1 ml of vehicle solution for 20 weeks were followed by the daily ingestions of 2.3 mg/kg b.w. D1 (group 4), weekly injections of 45 mg/kg b.w. 5-FU (group 5), and by concomitant daily ingestions of 2.3 mg/kg b.w. D1 and weekly injections of 45 mg/kg b.w. 5-FU (group 6), for 7 weeks (Fig. 2).

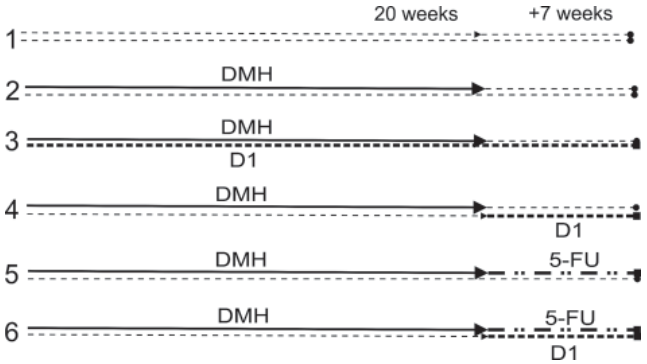

Fig. 2. Experimental protocol. Initially, 60 rats were divided into 6 groups (10 rats per group). Rats were treated with DMH (20 mg/kg) in 2–6 or the same volume of vehicle (0.1 ml) in 1, and with D1 (2.3 mg/kg) in 3 and the same volume of vehicle (0.1 ml) in others for 20 weeks. The intraperitoneal 5-FU injections (45 mg/kg) in 5–6 and D1 consumptions (2.3 mg/kg) in 3; 4; 6 for 7 weeks conducted as a treatment procedure

Tissue preparation. After the treatments, the rats were sacrificed by cervical dislocation, the abdomen was opened and the entire gastrointestinal tract was removed. The colon samples were fixed for 14 days in neutral saline solution containing 10% formalin. Then, they were embedded into paraffin and sliced into 5-μm sections, which were stained with hematoxylin-eosine-orange [16] and examined under the light microscope. The tumor number per animal (N_{tumor}) and the area of tumors were measured from the color images using the image processing and analysis program WCIF ImageJ. The tumor total area per animal (S_{tumor}) was calculated as a sum of the areas of all tumors in the colon. The mean tumor area represents the ratio of tumor total area over the total number of tumors. The colon samples with no tumors studied for detection of changes in the apparently healthy colon mucosa under the influence of the agents were examined under the light microscope. The mucosa thickness, the height and nuclei area of colonocytes and the area of the goblet cells were measured from the color microphotographs (magnification x400) using WCIF ImageJ software. The mitotic index (MI) in 300 colonic crypt epithelial cells per animal and crypt fission index (CFI) in 200 colon crypts per animal were calculated in the apparently healthy colon mucosa.

Statistical analysis was carried out using SPSS 17.0 software for Windows. One-way ANOVA followed by the post-hoc Bonferroni's test was employed to determine statistical significance. Correlation analysis was conducted using Spearman *rho* correlation test. $p < 0.05$ was considered statistically significant.

RESULTS

Visual inspection of the test animals detected tumors in the downstream section of the colon, mainly of exophytic type, which is consistent with published data [15]. Data for the tumor number, tumor mean and total area for all, exophytic (adenomas and hyperplastic polyps) and endophytic (adenocarcinomas) tumors showed that D1, 5-FU, and their combination D1+5-FU affect mainly the proliferation of exophytic tumors (Table 1).

Table 1. Tumor parameters for rats treated by D1, 5-FU and their combination under DMH-induced carcinogenesis (27 weeks) (Mean ± SEM, n = 10)

	DMH	DMH + D1 (27 weeks)	DMH + D1 (7 weeks)	DMH + 5-FU	DMH + D1 + 5-FU
			All		
Tumor number (N_{tumor})	9.56 ± 1.74	7.17 ± 1.70*	5.60 ± 1.27*	4.78 ± 1.44*	5.20 ± 1.71*
Tumor mean area, cm^2	0.17 ± 0.07	0.12 ± 0.06	0.17 ± 0.05	0.19 ± 0.13	0.14 ± 0.04
Tumor total area (S_{tumor}), cm^2	1.58 ± 0.65	0.86 ± 0.47*	0.94 ± 0.40*	0.91 ± 0.68*	0.73 ± 0.20*
			Exophytic Tumors		
Tumor number (N_{tumor})	9.00 ± 1.53	6.50 ± 2.23*	4.90 ± 1.38*	4.44 ± 1.42*	4.50 ± 1.41*
Tumor mean area, cm^2	0.12 ± 0.03	0.11 ± 0.06	0.10 ± 0.02	0.10 ± 0.04	0.12 ± 0.04
Tumor total area (S_{tumor}), cm^2	1.03 ± 0.28	0.56 ± 0.37*	0.49 ± 0.16*	0.46 ± 0.21*	0.49 ± 0.22*
			Endophytic Tumors		
Tumor number (N_{tumor})	0.67 ± 0.47	0.67 ± 0.67	0.70 ± 0.60	0.33 ± 0.31	0.70 ± 0.60
Tumor mean area, cm^2	0.82 ± 0.81	0.45 ± 0.44	0.64 ± 0.16	1.34 ± 1.26	0.29 ± 0.10
Tumor total area (S_{tumor}), cm^2	0.55 ± 0.53	0.3 ± 0.27	0.45 ± 0.42	0.45 ± 0.42	0.20 ± 0.18

Notes: *p<0.05 compared to DMH-treated rats.

The reduction of the total area by 46; 40; 42 and 54% comparing to DMH group (group 2), was observed in DMH+D1 27-week-treated rats (group 3), DMH+D1 7 weeks-treated rats (group 4), DMH+5-FU-treated rats (group 5) and DMH+D1+5-FU-treated rats (group 6), respectively (Table 1). The decrease in the tumor number by 25; 41; 50 and 46% comparing to DMH group (group 2), was found in DMH+D1 27-week-treated rats (group 4), DMH+D1 7 weeks-treated rats (group 3), DMH+5-FU-treated rats (group 5) and DMH+D1+5-FU-treated rats (group 6),

respectively. The reduction of the tumor mean area, as compared to the DMH group, in the above groups did not reach statistical significance. Thus, anti-tumor effects of D1 and 5-FU are similar. Notably, in DMH+D1 7 weeks- treated rats anti-tumor action is stronger comparing to DMH+D1 27 weeks group. In group 6, during the concomitant action of D1 and 5-FU, the effects of the agents are not summed up.

Light microscopy examination of the colon mucosa of all treated groups showed the inflammatory features in group DMH, manifested by infiltration of lymphocytes and histiocytes and blood capillary dilation. Crypts inlets sometimes were expanded possibly through hyperinflation from mucus check-valve mechanism. In groups DMH+D1 7 weeks and DMH+D1 27 weeks, inflammatory features were much less pronounced, as lower local lymphocytic-histiocytic aggregations in group 4 and no changes in group 3 were observed comparing to control. On the contrary, the inflammation was enhanced in group DMH+5-FU, as the infiltration of lymphocytes and histiocytes was more prevalent and the vessel dilations were more frequent. Damage of surface epithelium was also observed. Inflammatory features in colon mucosa of DMH+D1+5-FU animals were manifested more than in groups 3 and 4, but somewhat less than in group 5 (Fig. 3).

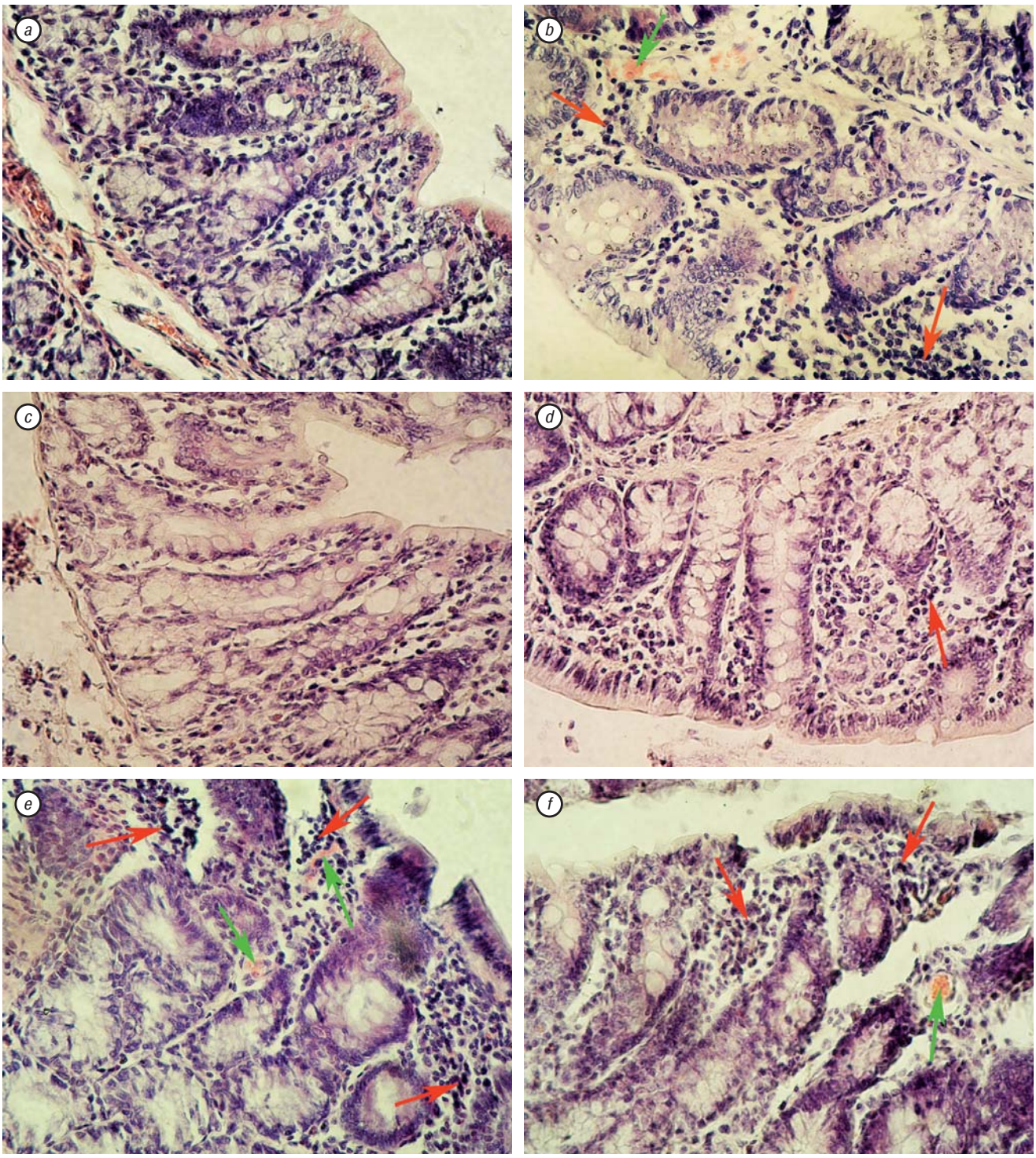

Fig. 3. Microphotographs of apparently healthy colon mucosa; hematoxylin-eosine-orange stain; magnification x400. *a* — group 1; *b* — group 2; *c* — group 3; *d* — group 4; *e* — group 5; *f* — group 6. Inflammatory features in groups 2; 5; 6 are manifested by infiltration of lymphocytes and histiocytes (red arrows) and blood capillary dilation (green arrows). Damage of surface epithelium appears in groups 5 and 6

Light microscopy also revealed a decrease of MI comparing to DMH group by 30.1%, 35.2% and 25.5% in DMH+D1 27 weeks-treated, DMH+5-FU-treated and DMH+D1+5-FU-treated groups, respectively (Table 2). Statistically significant changes of CFI between groups were not observed. Thus, a reduction of MI comparing to DMH group was observed in all test groups except of DMH+D1 7 weeks treated rats. Analysis of other mucosa parameters (Table 3) showed an increase of the mucosa thickness, the height of colonocytes and their nuclei area in animals of DMH group (group 2) compared to control. All these parameters were decreased by D1 acting for 7 weeks (group 4), whereas only the height of colonocytes and their nuclei area were reduced by D1 acting for 27 weeks (group 3). Moreover, in the rats of group 3, the mucosa thickness and the area of goblet cells were increased compared to both the control and DMH animals (group 2), which could be explained by increased mucus formation to provide protection against xenobiotic [16]. In DMH+5-FU animals (group 5), a decrease of the height of colonocytes, no changes in their nuclei area and an increase of the mucosa thickness compared to DMH ones (group 2) were found. Changes of mucosa morphometrial parameters of DMH+D1+5-FU-treated animals (group 6) could evidence that effects of D1 and 5-FU on the conditions of apparently healthy mucosa are summed up.

Table 2. Proliferative activity parameters of apparently healthy colonic mucosa for rats treated by D1, 5-FU and their combination under DMH-induced carcinogenesis (27 weeks) (Mean ± SEM, n = 10)

	MI, %	CFI, %
Control	4.25 ± 0.32	9.70 ±1.21
DMH	4.20 ± 0.26	9.84 ± 1.73
DMH+D1 (27 weeks)	3.03 ± 0.24*#	9.32 ± 1.22
DMH+D1 (7 weeks)	3.70 ± 0.35	8.34 ± 1.29
DMH+5-FU	2.87 ± 0.23*#	8.67 ± 1.07
DMH +D1+5-FU	3.18 ± 0.38*#	9.84 ± 1.92

Notes: *p<0.05 compared to control, #p<0.05 compared to DMH.

Table 3. Parameters of apparently healthy colon mucosa for rats treated by D1, 5-FU and their combination under DMH-induced carcinogenesis (27 weeks) (Mean ± SEM, n =10).

	Mucosa thickness, μm	Colonocytes height, μm	Colonocytes nuclei area, μm^2	Goblet cells area, μm^2
Control	210.64±9.62	16.75±0.87	14.93±1.22	107.08±11.3
DMH	238.56±11.64*	20.93±1.18*	18.59±1.03*	108.01±9.27
DMH + D1 (27 weeks)	257.97±11.64*#	18.60±0.88*#	14.60±1.08#	130.63±14.29*#
DMH + D1 (7 weeks)	209.48±9.35#	19.05±0.84*	15.80±0.81#	99.96±6.72
DMH + 5-FU	282.65±12.95*#	18.47±0.7*#	18.08±1.11*	100.91±6.78
DMH + D1 + 5-FU	239.93±11.44*	17.91±0.64*#	15.40±1.53#	97.12±6.17

Notes: *p<0.05 compared with control, #p<0.05 compared with DMH.

Correlation coefficients between MI and CFI in the apparently healthy colon mucosa were calculated as 0.13, -0.54 and 0.41 in all, DMH+D1-treated and DMH+5-FU+D1-treated rats, respectively. Correlation coefficients between N_{tumor} and S_{tumor} and characteristics of surrounding apparently healthy colon mucosa are presented in Table 4.

DISCUSSION

The rats from the DMH-induced cancer groups exhibited tumor localization mainly in the distal colon. The observed tumors were mainly exophytic, which is in good agreement with [15, 17, 18]. It was found that D1, 5-FU and their combination mainly affect the growth of exophytic tumors (adenomas and hyperplastic polyps) (Table 1), which can be explained by peculiarities of the development of tumors with different types of growth [17, 19, 20]. The main proliferation abnormality both in the hyperplastic polyps and adenomas appears to be an increase in the rate of crypt fission, a process that begins by basal bifurcation and is followed by longitudinal division of the crypt, with no changes in the proliferation (mitotic) index. However, in adenocarcinomas, proliferative index in the top zone of the crypt is higher compared to adenomas and adenomatous polyps.

D1 causes greater antitumor effect in case of the 7-week treatment compared to the 27-week one, which could be due to adaptation to D1 under its prolonged influence, but this certainly warrants more detailed further investigation.

Light microscopy examination of the apparently healthy colon mucosa of all treated groups showed less impact of D1 on the mucosa vascular bed and on proliferative activity in comparison with 5-FU, when both compounds act for the same duration. The effect of D1 is compounded with time exposure increasing (27 vs. 7 weeks), but remains less aggressive compared with 5-FU. It points out at the good toxicity profile of D1, which constitutes an important common feature of the targeted therapies safety [7].

Observed low correlation between MI and CFI of healthy mucosa reveals independence of crypt fission and cell proliferation processes, which agrees with the data of H.S. Park et al. [21, 22]. The correlation between MI and the tumor growth parameters was expected [17, 22, 23], as the latter is always characterized by the increased cell proliferation. The colon adenomas are characterized by more intensive cell proliferation assessed as a number of mitotic cells per one crypt. Thereat, proliferated cells are distributed along the whole length of the crypts, up to their upper parts, whereas, in normal mucosa, they reside only in the lower two-thirds of the crypts. Notably, adenocarcinomas are specifically characterized by intensive cell proliferation in the upper parts of the crypts and/or in the superficial epithelial layers.

Table 4. Spearman correlation coefficients (*rho*) between parameters of tumor growth (N_{tumor} and S_{tumor}) and surrounding colon mucosa (MI and CFI) for rats treated by D1, 5-FU and their combination under DMH-induced carcinogenesis (27 weeks)

		MI	CFI
All	N_{tumor}	0.41	
	S_{tumor}	0.45	
DMH	N_{tumor}	0.76	0.63
	S_{tumor}	0.77	
DMH+5-FU	N_{tumor}	0.5	
	S_{tumor}	-0.3	-0.73
DMH+D1	N_{tumor}		-0.35
	S_{tumor}		
DMH+D1+5-FU	N_{tumor}	-0.32	
	S_{tumor}		-0.41

Note: only statistically significant values are provided.

We analyzed the correlation between parameters of colon mucosa and tumor growth in experimental groups separately and revealed the difference in the

relationships of these parameters. In DMH-treated rats, MI strongly correlates with parameters of tumor growth and also CFI correlates with N_{tumor}, which is in agreement with data of H.S. Park et al. [21], W.M. Wong et al. [17], and S.J. Alrawi et al. [18], and supports the mechanism suggesting that the growth of adenomas and adenocarcinomas occurs mainly through the crypt fission [24].

Correlation of MI with N_{tumor} in DMH+5-FU-treated rats may indicate the mechanism of the tumor growth inhibition by 5-FU as an inhibition of cell proliferation [4]. Taking into account a decrease of MI and CFI under the influence of 5-FU, negative correlation of MI and CFI with S_{tumor} can be explained by the initial increase of crypt fission at the tumor growth initiation with its subsequent reduction to ensure the cell settlement of newly formed crypts under conditions of inhibited proliferation [23].

The decrease of N_{tumor} and S_{tumor} by D1 in DMH+D1-treated animals was observed, whereas no correlation between MI and parameters of tumor growth was found. The lack of correlation could be explained by suggestion that D1 mainly acts on transformed cells without affecting normal cells proliferation.

Positive correlation between MI and CFI, as well as negative correlations between MI and N_{tumor} and between CFI and S_{tumor} observed in DMH+5-FU+D1-treated rats indicate the difference in mechanisms of combined and separate action of D1 and 5-FU, which requires further investigation.

So, in conclusion, antitumor effects of D1 (5-amyno-4-(1,3-benzothyazol-2-yn)-1-(3-methoxyphenyl)-1,2-dihydro-3H-pyrrol-3-one) and 5-FU are similar, indicating the effectiveness of D1 as anti-neoplastic agent. D1 causes greater antitumor effect in case of 7 weeks treatment compared with 27 weeks treatment, which may be explained by adaptation to D1 under its prolonged influence. Impact of D1 on vascular bed and proliferative activity of surrounding healthy colon mucosa is gentlier compared with 5-FU. Changes in proliferative activity of surrounding healthy colon mucosa correlate with tumor growth parameters. The correlations between parameters of tumor growth and proliferative activity are different in the presence of either D1 or 5-FU indicating different mechanisms of action and different targets of these compounds.

Obtained results could be useful for better understanding of the processes occurring prior to malignant transformation of the colon epithelial cells and for their pharmaceutical correction.

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