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Współdziałanie proglumidu z 5-fluorouracylem na wzrost komórek raka jelita grubego Colon 38 in vitro

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Streszczenie

Rola gastryny i jej receptorów w rozwoju raka jelita grubego jest od dawna postulowana, lecz nadal pozostaje nie w pełni wyjaśniona. Z kolei skuteczność terapeutyczna fluorouracylu (FU), leku z wyboru w zaawansowanych postaciach raka jelita grubego, jest niewystarczająca, dlatego trwają poszukiwania czynników nasilających przeciwnowotworowe działanie FU. Ostatnio w naszym laboratorium wykazaliśmy synergistyczne działanie proglumidu, nieselektywnego blokera receptorów cholecystokininowo-gastrynowych, z FU w zahamowaniu proliferacji i nasileniu apoptozy w raku jelita grubego Colon 38 zaindukowanym u myszy in vivo.

Celem pracy było zbadanie bezpośredniego wpływu proglumidu (10^{-5}-10^{-10} M) stosowanego osobno lub łącznie z FU (25, 2,5 i 0,25 μg/ml) na proliferację komórek mysiego raka jelita grubego Colon 38 in vitro. Proliferację komórkową oznaczano w oparciu o zmodyfikowaną metodę kolorymetryczną Mosmanna.

Proglumid zastosowany osobno hamował proliferację komórkową w stężeniach 10^{-6}, 10^{-8}, 10^{-10} M. FU we wszystkich badanych stężeniach hamował proliferację komórek linii Colon 38 w porównaniu z grupą kontrolną, wykazując najsilniejsze działanie w stężeniach 2,5 i 25 μg/ml. Hamujące działanie FU w stężeniu 2,5 μg/ml okazało się nieznacznie silniejsze niż efekt 10-krotnie wyższego stężenia FU, dlatego do badań jego interakcji z proglumidem wybrano stężenie 2,5 μg/ml. Proglumid zastosowany łącznie z FU wykazywał synergistyczne działanie w zahamowaniu proliferacji komórkowej Colon 38 w stężeniach 10^{-8}, 10^{-9} i 10^{-10} M. Najsilniejszy efekt hamujący proliferację obserwowano w grupie inkubowanej z FU (2,5 μg/ml) i proglumidem 10^{-10} M.

Uzyskane wyniki wskazują na możliwość opracowania nowych schematów leczniczych w raku jelita grubego, co wymaga dalszych badań sprawdzających czy synergistyczne działanie FU z proglumidem występuje również w ludzkich rakach jelita grubego.

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Słowa kluczowe: *proglumid, fluorouracyl, proliferacja, rak jelita grubego Colon 38*

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The combine effects of proglumide and fluorouracil on the growth of murine Colon 38 cancer cells in vitro

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Abstract

The role of gastrins and their receptors in the pathogenesis of colon cancer has been discussed for many years but it still remains unresolved. Although fluorouracil (FU) remains to be reference chemotherapy for colon cancer, its efficacy is unsatisfactory. Recently, we have shown a synergistic effect of proglumide (a non-selective blocker of cholecystokinin-gastrin receptors) applied together with FU, on the proliferation and apoptosis of transplantable Colon 38 cancer cells in vivo.

The aim of this study was to examine direct effects of proglumide (10^{-5}-10^{-10} M) applied either alone or together with FU (0.25, 2.5 and 25 μg/ml) on the proliferation of murine Colon 38 cancer cells in vitro. Cell proliferation was assessed by the modified colorimetric Mosmann method.

Proglumide inhibited the proliferation of Colon 38 cells at the concentrations of 10^{-6}, 10^{-8} and 10^{-10} M. FU inhibited the proliferation of cancer cells in all studied concentrations, exerting the most profound antiproliferative effect at the concentrations of 2.5 and 25 μg/ml. Thus, the former concentration was chosen to study its interactions with proglumide. Proglumide applied together with FU exerted a synergistic effect on the inhibition of proliferation of Colon 38 cells at 10^{-8}, 10^{-9}, 10^{-10} M concentrations. The most profound inhibitory effect was observed in the group incubated with FU and 10^{-10} M of proglumide.

The obtained results indicate a possibility of new therapeutic options for colon cancer, but further studies are needed to elucidate, if the synergistic effect of FU and proglumide occurs also in the colon cancer in humans.

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Key words: *proglumide, fluorouracil, proliferation, Colon 38 cancer*

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Introduction

Colon cancer is a growing medical problem, especially in industrialized countries, where its incidence has increased systematically since the 1970s [1, 2]. Colon cancer is often diagnosed late, in advanced stages of the disease, when it is lethal. Until the mid-1990s, fluorouracil (FU) was the primary and the only chemotherapeutic agent, available for the treatment of advanced-stages of local and metastatic colorectal cancers, but its efficacy is unsatisfactory [3]. During the last decade, five new drugs have been approved for treatment of metastatic colorectal cancer. Three of them are cytostatic agents: irinotecan, oxaliplatin and capecitabine, and two of them include non-cytotoxic monoclonal antibodies (approved by FDA in 2004), targeting VEGF (bevacizumab) and EGF receptor (cetuximab) [4]. Despite these new possibilities, colorectal cancer treatment still remains only palliative with no proofs for any increase of cure rates. These a.m. non-cytotoxic agents demonstrate a significant antitumor activity in patients with advanced colon cancer, either alone (cetuximab in patients with EGF-R positive and irinotecan-refrectory tumors) or in combination with cytotoxic drugs [5, 6]. Moreover, an addition of one of these non-cytotoxic agents to fluorouracil- or irinotecan-based chemotherapy improves the survival among patients with metastatic cancer [5, 6]. The observed clinical advantages of these new non-cytotoxic drugs over traditional chemotherapy allow expecting these targeted drugs to become gold means and standard therapies for colorectal cancer within a short period of time [4].

Another target in colorectal cancer seems to be gastrin receptors. The role of gastrins and their receptors in the development of colon cancer has been postulated for several years but it still remains uncertain. It has been shown that: gastrin levels are

higher in patients with colon cancer than in normal people [7]; the presence of gastrin, its precursor, gastrin mRNA and gastrin-cholecystokin receptors B in colon cancer tissues have been found [8, 9, 10], however, opposite results also exist [11, 12]. Moreover, trophic effects of gastrin or pentagastrin (gastrin analog) [13, 14] and antiproliferative and/or proapoptotic effects of proglumide (a non-specific antagonist of gastrin/cholecystokinin receptors) [13, 15, 16] have been demonstrated on colon cancer growth but there have also been contradictory reports [12]. Are gastrins to be regarded as promoters of colorectal cancer? But the recent data speak in favor of this conception. In a large prospective case-control study (among 128,992 subscribers), published in 1998 [17], the authors concluded that hypergastrinemia was associated with an increased risk of colon cancer in about 9% of cases. Moreover, in the recent years, the hypothesis, concerning the role of gastrins in colon carcinogenesis, has changed on the basis of the results from transgenic animals, either lacking or overexpressing various forms of gastrins [18, 19, 20]. Most of recent studies have shown that neither matured form of gastrin (amidated gastrin) nor classical gastrin /CCK-B receptors are involved in colon carcinogenesis, but its less processed forms (glycine-extended gastrin and progastrin) and putative gastrin-glycine receptors seem to be the major players. In the excellently designed study, glycine-extended gastrin (G-Gly), but not matured gastrin, increased the invasiveness of colon cancer cells, what was inhibited via non-selective gastrin/CCK receptor antagonists (proglumide and benzotript), but not via specific gastrin/CCK-B antagonist (YM022) [21]. The other authors found that transgenic mice with pharmacologic levels of progastrin exhibited increased susceptibility to colon carcinogenesis in response to carcinogen (azoxymethane) [18, 20], whereas gastrin knockout mice were also more susceptible to carcinogen than the wild-type mice [19]. These data suggest even a protective role of amidated gastrin in colon carcinogenesis and a potent co-carcinogenic role of its less processed forms, termed nonamidated gastrins (progastrin and glycine-extended gastrin). In the light of these data, the earlier discrepancies in the literature, concerning the role of gastrins in colon carcinogenesis, seem to be understandable.

Recently, in our laboratories, two separate experiments were performed (short and long lasting) with pentagastrin and proglumide (effective in inhibiting action evoked via putative G-Gly receptors), given either separately or together with FU on the growth of the transplantable Colon 38 cancer in mice. We found that proglumide alone induced apoptosis of tumor cells, in both the short- and the long-term studies and, administered jointly with FU, it evoked even a synergistic effect on the inhibition of cell proliferation and induction of apoptosis in the long term study [13, 16]. Those data suggest both direct or indirect effects of proglumide on Colon 38 cancer.

Thus, the aim of the present study was to examine the direct effects of proglumide at various concentrations, applied either alone or together with FU, on the proliferation of murine Colon 38 cancer cells in vitro.

Material and methods

Murine Colon 38 cancer cells were used in the study. The cells were cultured in a culture flask (Nunc Eas Y flask 25cm^2, NUNC) in the presence of RPMI 1640 medium (Sigma), supplemented with: 25 mM Hepes buffer (Sigma), 4 mM L-glutamine (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin solution (Sigma), 2g/l sodium bicarbonate (Sigma) and 5% fetal calf serum (FCS, Biochrom) (complete medium). The cells were routinely cultured in a humidified incubator at 37^{o}C with 5% carbon dioxide. Before confluency, the cells were harvested every 3-4 days in the presence of preheated (37^{o}C) trypsin-EDTA at concentration of 0.05 and 0.02% respectively, in Hanks-balanced salt solution (Trypsin-EDTA, Sigma). Thereafter, the cells were collected, rinsed three times in culture medium, centrifuged and seeded in culture flask ($2x10^5$ cells/5 ml medium) for subsequent 4 days. After one of the following trypsinization procedures the cells were suspended in complete medium in amount of $4x10^5$ cells/ml. Fifty µl aliquots of this suspension ($20x10^3$ cells) was seeded into each well of the culture plate (96 Cell Culture Cluster Dish, Nunclon MicroWell Plates, NUNC) and preincubated for 24 hours. Then, the cells were cultured for further 24 hours in the presence of various concentrations of the examined substances (fluorouracil and proglumide) applied either alone or jointly. Three separate cultures were performed. In culture I the effects of fluorouracil (**FU**, Fluoro-uracil, Roche) were examined at concentrations of 25, 2.5 and 0.25 µg/ml; the control groups were incubated in complete medium only. In culture II, the effects of various concentrations of proglumide (**P**, Proglumide, Sigma) (10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}M) were investigated; the control groups were cultured in presence of proglumide solvent (ethanol with complete medium; ethanol concentration was $6.6x10^{-3}$ vol% as in the wells with the highest concentrations of proglumide). In culture III the effects of various concentrations of proglumide (10^{-8}, 10^{-9} i 10^{-10}M), applied either alone or together with fluorouracil, at the concentration of 2.5 µg/ml, were studied; the control groups for fluorouracil ($\mathbf{C_m}$) were cultured in presence of complete medium only, for proglumide ($\mathbf{C_e}$) in the presence of complete medium with ethanol (ethanol concentration $7.4x10^{-6}$vol%). The

cell proliferation was assessed by the colorimetric Mosmann method, using the EZ4Y kit (Easy for You, The 4th Generation Non Radioactive Cell Proliferation & Cytotoxity Assay, Biomedica Gruppe, Austria, Bellco Biomedica Poland). This method is based on the transformation of tetrazolium salt into coloured soluble formazan via mitochondrial enzymes, what correlates well with cell proliferation and viability. The intensity of reaction was estimated via measurement of optical density (OD), using an ELISA reader (λ=450nm). The data were statistically analyzed by ANOVA and the significance of differences between means was determined by LSD (Least Significant Differences). P<0.05 was considered as the borderline of statistic significance.

Results

The obtained results are present on Figures 1-3. We designed culture I as having the most effective antiproliferative concentration of fluorouracil (FU) on Colon 38 cancer cells in vitro. We found that all the examined concentrations of FU inhibited the proliferation of Colon 38 cancer cells (Fig. 1). The most profound antiproliferative effect of FU was obtained in the following two concentrations: 2.5 and 25 μg/ml. The inhibitory effect of FU, at the concentration of 2.5 μg/ml, was slightly stronger than the effect of a 10 times higher concentration of FU (25 μg/ml), thus we chose the former concentration to study the interaction between FU and proglumide. Proglumide, applied alone, inhibited the proliferation of Colon 38 cells at the concentrations of 10^{-6}, 10^{-8} and 10^{-10} M (Fig. 2, 3). The other examined concentrations of proglumide (10^{-5}, 10^{-7}, 10^{-9} M) were insufficient in inhibiting Colon 38 cancer cell proliferation effectively. Proglumide, applied together with FU (2.5 μg/ml), exerted a synergistic effect (stronger than the effect of FU or proglumide applied alone) on the inhibition of cell proliferation of Colon 38 cancer in all the examined concentrations (10^{-8}, 10^{-9}, 10^{-10} M) (Fig. 3). Even at the concentration of 10^{-9} M, which was ineffective in the inhibition of cell proliferation, proglumide, applied together with FU, enhanced the antiproliferogenic effect of the latter. The most profound inhibitory effect was observed in the group incubated in the presence of FU (2.5 μg/ml) and proglumide at the lowest of the examined concentrations, e.g., 10^{-10} M (Fig. 3).

Discussion

The data, obtained by us, indicate that proglumide caused a slight but statistically significant, direct inhibitory effect on the proliferationof Colon 38 cancer. In this study, proglumide, applied alone, inhibited cell growth of Colon 38 cancer in vitro at

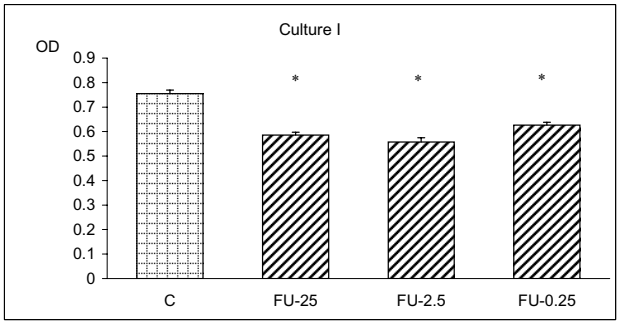

Fig. 1. *Effects of various concentrations of fluorouracil (FU, 0.25; 2.5 and 25 μg/ml) on the proliferation of Colon 38 cancer cells. C- control, OD-optical density, X±SEM, *P<0.001 vs C.*

Ryc. 1. *Wpływ różnych stężeń 5-fluorouracylu (FU; 0,25; 2,5 and 25 μg/ml) na proliferację komórkową linii Colon 38. C- kontrola, OD-optical density, X±SEM, *P<0,001 vs C.*

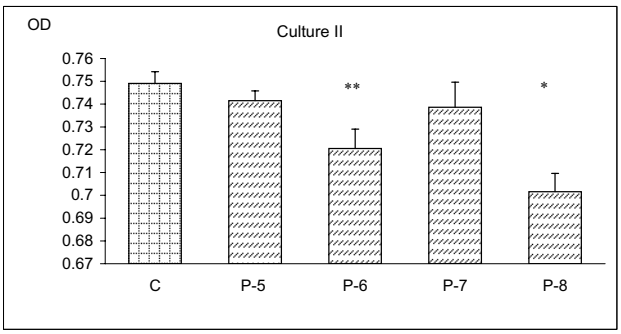

Fig. 2. *Effects of various concentrations of proglumide (P) on the proliferation of Colon 38 cancer cells. C-control, OD-optical density, X±SEM, *P<0.001 vs C, **P<0.01 vs C.*

Ryc. 2. *Wpływ różnych stężeń proglumidu (P) na proliferację komórkową linii Colon 38. C-kontrola, OD-optical density, X±SEM, *P<0.001 vs C, **P<0.01 vs C.*

the concentration of 10^{-6}, 10^{-8}, 10^{-10} M, but it did not change the proliferation at other examined concentrations, such as: 10^{-5}, 10^{-7}, 10^{-9} M. The explanation of these divergent effects of the changed concentrations of proglumide might be connected with the existence of three receptor subtypes, which mediates the effect of this drug on Colon 38 cancer cells. This speculation is based on the fact that proglumide, the non-selective antagonist of gastrin/CCK receptors (existing in two classical subtypes: gastrin/CCK-A and B receptors, presently called CCK1 and CCK2 respectively), seems to block also the putative gastrin-Gly (G-Gly) receptors, through which the less processed forms of gastrin exert their growth effect [21]. Another explanation of this changing (fluctuating) effect of proglumide may depend on the too short period of time of the culture; it lasted only 24 hours. Perhaps, the culture should have lasted much longer to observe full inhibitory effects

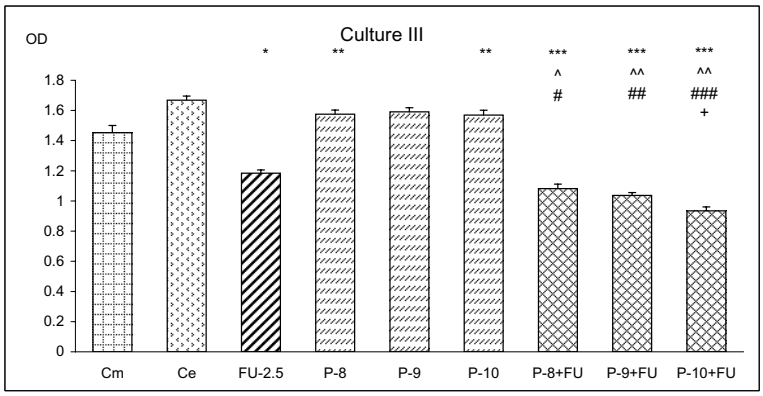

Fig. 3. *Effects of proglumide (P), 5-fluorouracil (FU) and of the combination of proglumide and 5-fluorouracil, in concentration of 2.5 µg/ml, on the proliferation of Colon 38 cancer cells. C_m-control for fluorouracil (with complete medium only), C_e-control forproglumide (complete medium with ethanol), OD-optical density, X±SEM, *P<0.001 vs C_m, **P<0.05 vs C_e, ***P<0.001 vs C_m and C_e, ^P<0.05 vs FU-2.5, ^^P<0.001 vs FU-2.5, #P<0.001 vs P-8, ##P<0.001 vs P-9, ###P<0.001 vs P-10, +P<0.01 vs P-8FU and vs P-9FU.*

Ryc. 3. *Wpływ proglumidu (P), 5-fluorouracylu (FU) oraz łącznego zastosowania proglumidu i fluorouracylu w stężeniu 2,5µg/ml na proliferację komórkową linii Colon 38. C_m-kontrola dla fluorouracylu (medium pełne), C_e-kontrola dla proglumidu (medium pełne i etanol), OD-optical density, X±SEM, *P<0,001 vs C_m, **P<0,05 vs C_e, ***P<0,001 vs C_m i C_e, ^P<0.05 vs FU-2,5; ^^P<0,001 vs FU-2,5; #P<0,001 vs P-8; ##P<0,001 vs P-9; ###P<0,001 vs P-10; +P<0,01 vs P-8FU i vs P-9FU.*

of proglumide. On the basis of the obtained data, we hypothesize that a direct antiproliferative effect of proglumide on Colon 38 cancer cells is caused via the interruption of postulated autocrine loop, consisting of nonamidated gastrins (probably secreted by the examined Colon 38 cancer cells) and their putative gastrin-Gly receptors.

The antiproliferogenic effect of proglumide, found in this study, is in accordance with our own data showing proapoptotic effect of proglumide on Colon 38 cancer cells in vivo, in both the short-term and the long-term study [13, 16]. It is worth recalling, that the Mosmann method, used in the present study, measures the metabolic status of the cells, which positively correlates with the number of proliferating cells and negatively with the number of apoptotic cells. Our results are also compatible with the data obtained by other authors, who have showed that proglumide inhibits the growth of colon cancer cells, both in vivo and in vitro and enhances survival in tumor-bearing mice [15, 22].

Despite these encouraging data concerning the beneficial effects of proglumide on colon cancer growth and the survival of animals, the chances of the possible use of this drug (introduced into routine medical practice) in the treatment of colon cancer patients have not been evaluated, since some reports show that matured forms of gastrin are not involved in colorectal carcinogenesis [23, 24]. It has been postulated that neither amidated gastrin nor classical gastrin/CCK receptors are involved in colorectal carcinogenesis. The problem seems to be even more complicated because other authors have suggested that colon cancers, originating from different part of the colon, seem to have different pathogenesis [25].

Since proglumide has been shown to inhibit a proliferogenic action of nonamidated (intermediate, less processed) forms of gastrins on colon cancer cells via blocking the putative gastrin-Gly receptors, the renaissance of this drug starts again in the study, concerning colon cancer [21].

In the present study, we checked also combined effects of FU and proglumide on the growth of Colon 38 cancer cells. We found that proglumide in all the examined concentrations, applied jointly with FU, evoked a synergistic antiproliferogenic effect on Colon 38 cancer cells. This beneficial combined effect of proglumide and FU remains in accordance with our own data, showing that these two drugs caused the strongest antiproliferative and proapoptotic effect on Colon 38 cancer, transplantable in mice, in comparison to the effect, evoked by each of these substances, when given separately [13].

On the basis of these findings, we hypothesize that a combined administration of FU and proglumide in the treatment of colon cancer patients would improve the effectiveness of FU-based chemotherapy. Further studies are needed to elucidate whether this suggestion is worth clinical applications.

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