Applied Cell Biology

ISSN : 2320-1991 Online
ISSN : 2320-1983 Print
Volume 14 Issue 2
DOI: https://dx.doi.org/10.53043/2320-1991.acb14004

Research Article
Appl Cell Biol, 14(2), 2026 [13-20]
Published: July 10, 2026

Effects of Sanomucin on Intestinal Epithelial Cells and Inflammation-Related Cellular Responses *in Vitro*

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ABSTRACT

Background: Sanomucin by Helixor is a dietary supplement containing plant enzymes, lentil extract, vitamins, and selenium; According to authorized health-claims, biotin helps to maintain normal mucous membranes, while selenium and vitamin C support the body's normal functions. Sanomucin might also be used during tumor and demanding therapies. Sanomucin is no medication against tumors and does not substitute an oncological therapy.

Experimental: By using cultured organ-specific cells (intestinal epithelial cells, IPEC-J2) and functional neutrophils (differentiated HL-60 cells), the effect of Sanomucin concentrations (100 to 500 µg/mL) on cell metabolism, regeneration, cell viability after oxidative stress and inflammation was investigated. The tested Sanomucin concentrations correspond to theoretically calculated exposure-related concentrations in the blood fluid after application of the recommended daily intake.

Results: Sanomucin stimulated the basal cell metabolism of intestinal epithelial cells in a dose-dependent and significant manner compared to untreated cells. At a concentration of 100 µg/mL the stimulation was 8.7 ± 7.0 %, at 250 µg/mL 16.0 ± 4.7 % and at 500 µg/mL 41.1 ± 13.3 % (n = 3). Related to the residual cell-free area at the end of the experiment, Sanomucin significantly stimulated epithelial cell regeneration by 41.1 ± 17.4 % at 100 µg/mL compared to the untreated controls, and by 28.2 ± 11.2 % at 250 µg/mL (n = 3). Hydrogen peroxide-induced oxidative stress caused a decrease in cell viability/cell survival at concentrations > 1 mM in the culture medium. A significant increase in cell survival after application of Sanomucin by 23.7 ± 7.2 % at 100 µg/mL, by 39.3 ± 13.3 % at 250 µg/mL, and by 59.7 ± 9.4 % at 500 µg/mL was observed (n = 5). Finally, Sanomucin reduced the basal cell metabolism as well as radical generation by functional neutrophils in comparison to controls by 22.9 ± 7.1/22.9 ± 6.1 % at 100 µg/mL, 34.8 ± 10.1/35.4 ± 10.2 % at 250 µg/mL and 36.1 ± 11.4/36.1 ± 9.7 % at 500 µg/mL (n = 3). All data given are mean values ± standard deviations with a $p \leq 0.01$ by using the two-tailed Mann-Wilcoxon-Whitney test).

Conclusions: The results presented here suggest that Sanomucin may contribute to supporting cellular responses associated with mucosal integrity *in vitro.*

Abbreviations: IPEC-J2: Intestinal epithelial cells; HL-60: Human promyelocytes

Keywords

Dietary supplement
Mucosal barrier
Intestinal health
Intestinal barrier
Regeneration
Inflammation
Oxidative stress
Biotin
Selenium
Lentil extract
Vitamin C
IPEC-J2 cells
HL-60 cells
Cell culture

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INTRODUCTION

Healthy mucosal cells, which form a protective layer in many parts of the body, divide very rapidly. Tumor cells also share this characteristic. Since tumor therapies have rapidly dividing cells as the target, this means that chemotherapy attacks not only tumor cells but also mucosal cells, which can cause inflammation [1-4]. Since mucous membranes are found in many sensory organs, mucosal inflammation (mucositis) often limits important aspects of patients.

Sanomucin is a dietary supplement containing plant enzymes, lentil extract, vitamins, and selenium including Biotin and vitamin C. It has been developed as a dietary supplement intended to support nutritional status during demanding physiological conditions.

Polyphenols and further bioactive compounds in lentils have anti-microbial properties and act as antioxidants with protective functions against oxidative stress [5,6]. Selenium and its health effects are somewhat contradictory in human studies. Both, selenium deficiency and excess, have been associated with adverse health effects although conditions involving oxidative stress and inflammation have shown benefits of a higher selenium status [7,8]. Biotin acts as a cofactor for biotin-dependent carboxylases and is essential for the survival of all living organisms. Since mammals cannot produce biotin on their own, they must obtain it from their diet. It has been shown that biotin not only acts as a coenzyme in metabolism but also modulates intracellular signaling pathways and the expression of enzymes involved in metabolic processes. Biotin has also been linked to various inflammatory diseases [9,10]. Vitamin C is essential for connective tissue, wound healing, antioxidant protection [11,12] and is also used in anticancer therapies [13]. Moreover, the proteolytic enzymes bromelain and papain have been shown to possess a variety of biological effects. Besides them are also anti-inflammatory properties [14,15].

This study aimed to evaluate the effects of Sanomucin on epithelial metabolism, regeneration, oxidative stress resistance, and inflammatory responses using *in vitro* cell models.

MATERIALS AND METHODS

Test substance

For the studies, Sanomucin, item no. 3001890, batch 9071325, expiry date: 12/2027, manufactured by Helixor Heilmittel GmbH, D-72348 Rosenfeld, Germany, was used. According to the manufacturer, two enteric-coated capsules should be taken once a day before or after a meal without chewing, and with plenty of liquid. The daily dosage of Sanomucin contains 875 mg of active ingredients. If this amount is distributed initially over 1,500 mL of small intestinal fluid, the concentration is approximately 580 µg/mL. If this dose is distributed over 3,500 mL of blood liquid (assuming a theoretical bioavailability of 100%; cellular blood components are not taken into account), the concentration in the blood is approximately 250 µg/mL. Therefore, the following test concentrations of Sanomucin were selected for the *in vitro* studies: 0 – 100 – 250 – 500 µg/mL. Here, "0" represents the solvent control (= phosphate buffered saline) without active ingredients.

Sanomucin was solubilized as a highly concentrated stock solution (20 mg/mL) in phosphate buffered saline and the respective 10-fold concentrated stock solutions for the tests were then prepared by further dilution with phosphate buffered saline. The experiments were performed over a period of several weeks.

Cell culture

The investigations were performed with two different cell types: (1) Porcine intestinal epithelial cells (IPEC-J2 cells; ACC-701; Leibniz Institut, DSMZ, Braunschweig, Germany) were taken for the experiments at passages 21 to 28. The cells were routinely grown in a mixture (1:1) of Dulbecco's Modification of Eagle's Medium and Ham's F12 supplemented with 10 % growth mixture and standard amounts of antibiotics. The cells were routinely cultivated as mass cultures and were regularly subcultured twice a week with fresh culture medium. (2) Human promyelocytes (HL-60 cells; ACC 3; Leibniz Institute, DSMZ, Braunschweig, Germany) which were differentiated to functional neutrophils able to undergo an oxidative burst upon phorbol ester stimulation similar to primary and freshly isolated neutrophils [16-18]. HL-60 cells were cultivated as suspended mass cultures in RPMI 1640 medium supplemented with 10 % growth mixture and standard amounts of antibiotics and were regularly subcultured twice a week with fresh culture medium. HL-60 cells were taken for the experiments at passages 11 to 16. Basically, cell cultivation was performed in an incubator (Heraeus function line BB 16) at 37 °C in a humid atmosphere of 5 % CO_2 and 95 % air.

Examination of cell metabolism

IPEC-J2 cells were seeded into 96-well culture plates (200 µL culture medium/well) at a cell density of 100,000 cells/well and incubated for 48 hours until the cells had completely adhered and restored their metabolism. Thereafter, cells were incubated in a reaction mixture consisting of 160 µL/well of

culture medium, 20 µL of the Sanomucin stock solutions and 20 µL/well of WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt; Sigma-Aldrich, Deisenhofen, Germany). The WST-1 assay does not directly measure the number of living cells, but rather their metabolic dehydrogenases activity [19-21]. The optical density was measured as a difference measurement ΔOD = 450 – 690 nm at definite time points with an Elisareader (BioTek ELx808 & Gen 5 version 3.00) and analyzed using Microsoft Excel. Three independent experiments (n = 3) with biological replicates for each Sanomucin concentration were performed.

Examination of cell regeneration

Intestinal epithelial cells were seeded at a density of 200,000 cells/mL into the four individual compartments of a silicone 4 well-culture insert (ibidi, Gräfelfing, Germany). The single compartments of the inserts are separated by 500 µm thick silicone bars. Due to the special adhesion area, each insert adhered firmly to the bottom of a culture dish and formed a distinct cell-free area, which the cells colonized by migration and proliferation after removal of the silicone frame. Upon reaching confluency within 48 hours after cell seeding and directly after removal of the insert, the different concentrations of Sanomucin were added to the culture medium and remained there until the end of the experiment. The control cultures were handled simultaneously in the same manner with the addition of the same amount of solvent. After 12 hours of continuous incubation, cell cultures were washed with phosphate-buffered saline, fixed with methanol, stained with Giemsa's methylene blue solution (Sigma-Aldrich, Deisenhofen, Germany) and were air-dried. Micrographs documenting the residual cell-free area were done at different locations for each sample. A total of 4 measurements of the remaining cell-free area was carried out for each independent test series. IKOSA AI software with artificial intelligence (Kolaido, Altenrhein, Switzerland) was used to examine and calculate the residual cell-free area for the treated cell cultures in comparison to untreated controls. Three independent experiments (n = 3) with biological replicates for each test concentration were performed.

Examination of hydrogen peroxide-induced oxidative stress

Cells were seeded into 96-well culture plates (200 µL culture medium/well) at three different cell densities (200,000, 100,000 and 50,000 cells/well) and incubated for 24 hours until the cells had completely adhered and restored their metabolism. Then, cells were treated with hydrogen peroxide concentrations ranging from 0.5 to 2 mM in the culture medium together with different concentrations of Sanomucin for the duration of the experiment [22-25]. After 24 hours of hydrogen peroxide exposure, cell viability was examined by incubation of the cells to a reaction mixture consisting of culture medium and the water-soluble tetrazolium dye XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; Xenometrix, Allschwil, Switzerland). The cleavage of the dye is used as a colorimetric method for the quantification of cell viability [26-28]. The optical density was measured as a difference measurement ΔOD = 450 – 690 nm at definite time points with an Elisareader (BioTek ELx808 & Gen 5 version 3.00) and analyzed using Microsoft Excel. Five independent experiments (n = 5) with biological replicates for each Sanomucin concentration were performed.

In addition, cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole; Hello Bio, Dunshaughlin, County Meath, Republic of Ireland) at the end of the experiment according to the manufacturer's protocol. DAPI is a fluorescent dye that binds strongly to DNA, especially A-T rich regions and allows to visualize apoptotic cells [29,30].

Examination of anti-inflammatory potential

Promyelocytes were cultivated as suspended mass cultures in cell culture flasks with a vented cap (75 cm^2 growth area; TPP, Switzerland). The cultured promyelocytes were differentiated into functional neutrophils by adding 1.5 vol% dimethyl sulfoxide for 6 days. Finally, the cells were harvested by centrifugation (190 x g for 6 minutes) and were repeatedly washed in phosphate buffered saline with calcium and magnesium and centrifugation steps. Cells were resuspended in phosphate buffered saline with calcium and magnesium containing 10 mM glucose. 40 µL of the cell suspension aliquots were pipetted to a reaction mixture containing Sanomucin, glucose solution, the tetrazolium dye WST-1 (Sigma-Aldrich, Deisenhofen, Germany) and phorbol-12-myristate-13-acetate (Sigma-Aldrich, Deisenhofen, Germany) for induction of an oxidative burst [31-34]. Cells without addition of the phorbol ester served as internal controls reflecting the basal cell metabolism without stimulation. The generated reactive superoxide anion radicals in the reaction mixture caused the cleavage and color change of the dye. The amount of superoxide anion radicals present in the reaction mixture was directly related to this color change. The change in optical density was recorded at various time points up to 40 minutes as a differential measurement ΔOD = 450-690 nm by an Elisareader (BioTek ELx 808 with software Gen 5 version 3.00) and calculated with Microsoft Excel. Three independent experiments (n = 3) with biological replicates for each experiment were performed.

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STATISTICAL ANALYSIS

Data are given as mean values ± standard deviations. Biological replicates were averaged within each independent experiment before statistical testing. Statistical analyses was done using the parameter-free two-tailed Wilcoxon-Mann-Whitney rank sum test and significance was determined at the 1 % ($p \leq 0.01$) level.

RESULTS

Cell metabolism

The basal cell metabolism of intestinal epithelial cells was stimulated in a dose-dependent manner. At a Sanomucin concentration of 100 µg/mL the stimulation compared to the control cells with the same amount of solvent was 8.7 ± 7.0 %, at 250 µg/mL 16.0 ± 4.7 % ($p \leq 0.01$) and at 500 µg/mL 41.1 ± 13.3 % ($p \leq 0.01$).

Cell regeneration

Related to the residual cell-free area at the end of the experiment, Sanomucin stimulated epithelial cell regeneration by 41.1 ± 17.4 % at 100 µg/mL compared to the untreated controls, and by 28.2 ± 11.2 % at 250 µg/mL. The difference of both measurement data to the corresponding controls was statistically significant ($p \leq 0.01$). For further morphological information, see the micrographs in Figure 1.

Hydrogen peroxide-induced oxidative stress

As expected, the viability of the cells decreased with hydrogen peroxide concentrations > 1 mM in the culture medium. However, we observed a significant increase in cell survival after application of Sanomucin by 23.7 ± 7.2 % at 100 µg/mL, by 39.3 ± 13.3 % at 250 µg/mL, and by 59.7 ± 9.4 % at 500 µg/mL. The differences were significant ($p \leq 0.01$) compared to untreated solvent controls (Figure 2).

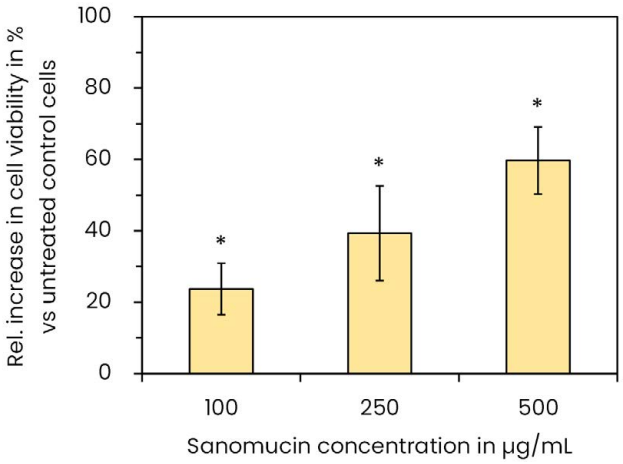

Figure 2: Graphical presentation of the relative increase of cell viability/cell survival after oxidative stress due to the addition of hydrogen peroxide at concentrations > 1 mM to the culture medium. All Sanomucin concentrations show a significant difference to untreated controls (*$p \leq 0.01$; two-tailed Wilcoxon-Mann-Whitney test). Data represent mean values ± standard deviations of five independent experiments (n = 5).

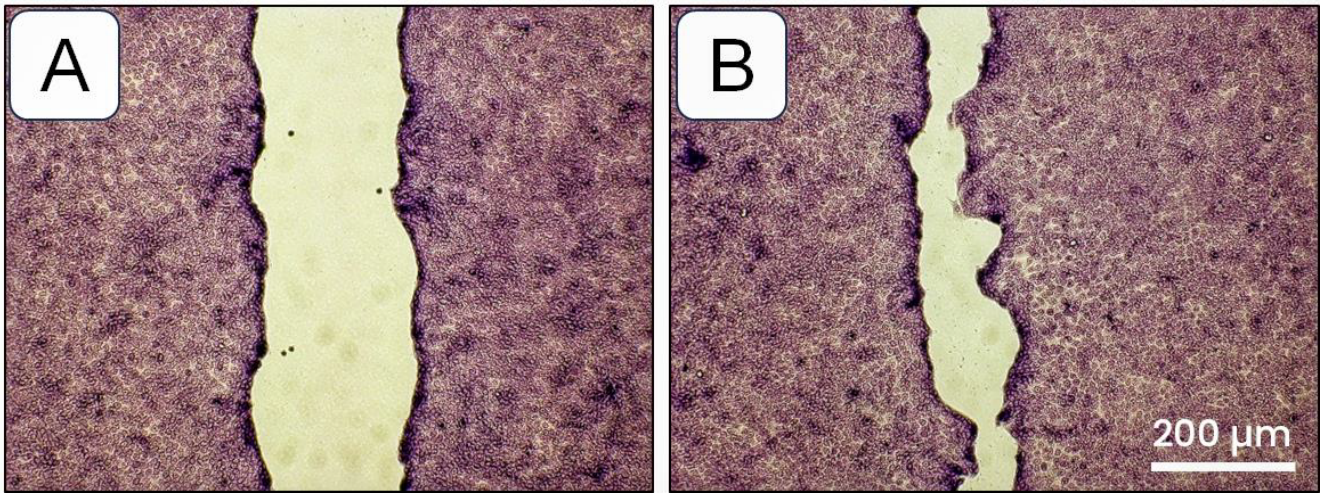

Figure 1: Representative micrographic documentation of the residual cell-free area after 12 hours of regeneration for untreated intestinal epithelial cells (A) and cells which were treated with 100 µg/mL Sanomucin for the duration of the experiment (B). Olympus IX50 inverted microscope with Olympus Planachromat 10x and Olympus E-20 at 5 megapixel. Brightfield illumination of stained samples.

Although not yet quantified, the number of apoptotic cells appeared increased in samples without Sanomucin protection (Figure 3).

Anti-inflammatory potential

The application of Sanomucin resulted in a dose-dependent decrease of the basal cell metabolism of functional neutrophils compared to untreated solvent controls by 22.9 ± 7.1 % at 100 µg/mL, 34.8 ± 10.1 % at 250 µg/mL and 36.1 ± 11.4 % at 500 µg/mL (all values with $p \leq 0.01$). Simultaneously, the generation of superoxide anion radicals was decreased by 22.9 ± 6.1 % at 100 µg/mL, 35.4 ± 10.2 % at 250 µg/mL, and 36.1 ± 9.7 % at 500 µg/mL (all values with $p \leq 0.01$). This effect is depicted in Figure 4.

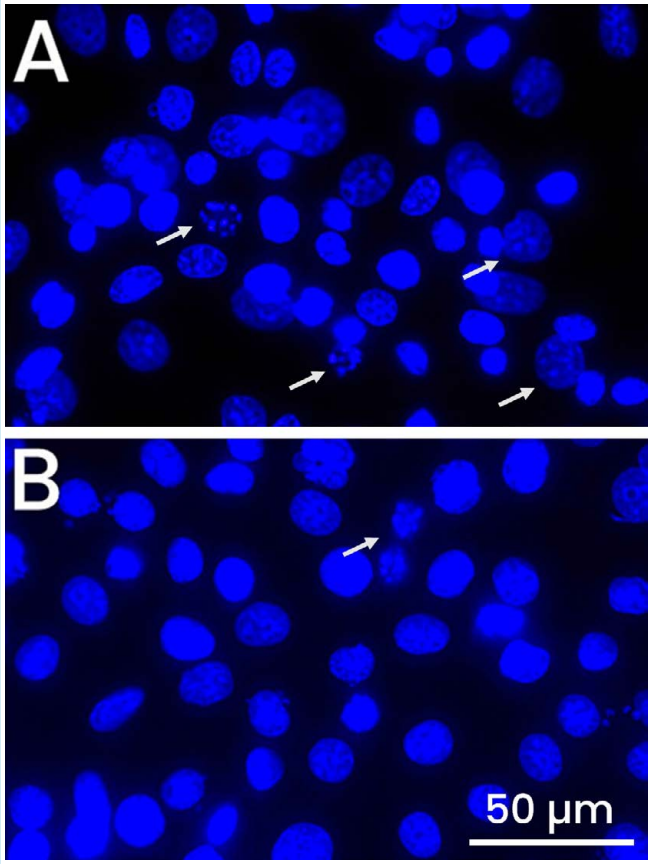

Figure 3: DAPI staining of cell nuclei after 24 hours of oxidative stress caused by the addition of 1.5 mM hydrogen peroxide. Apoptosis was confirmed by DAPI staining which showed apparent changes in the nuclear morphology such as chromatin condensation and nuclear fragmentation (white arrows). (A) Cells without treatment and (B) cells treated with 250 µg/mL Sanomucin. Keyence BZ-X1000 with Planapochromat 40x at UV illumination.

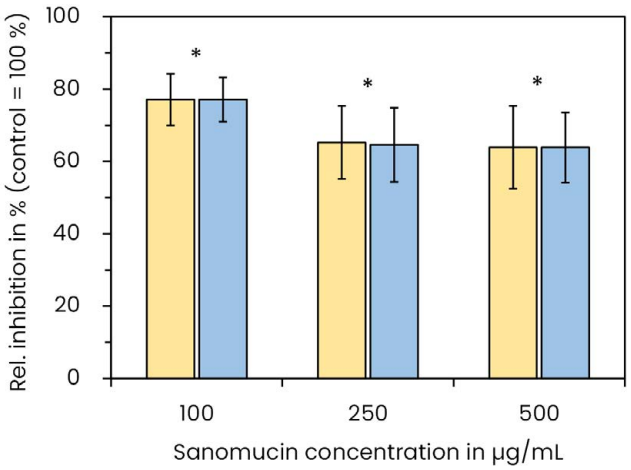

Figure 4: Graphical presentation of the relative basal metabolism of functional neutrophils (blue columns) and the superoxide anion generation after stimulation with a phorbol ester (yellow columns). Values refer to untreated control cells which are set as 100 %. All Sanomucin concentrations show a significant difference to untreated controls (*$p \leq 0.01$; two-tailed Wilcoxon-Mann-Whitney test). Data represent mean values ± standard deviations of three independent experiments (n = 3).

DISCUSSION

The IPEC-J2 cell line was chosen for the experiments, because it is "unique and derived from the small intestine of a pig and is neither transformed nor tumorigenic in nature. IPEC-J2 cells mimic the human physiology more closely than any other cell line of non-human origin" [35]. The cells were originally isolated in 1989 by Helen Berschneider at the University of North Carolina [36]. The advantage of the IPEC-J2 cell line as an in vitro model originates from its morphological and functional similarities with intestinal epithelial cells *in vivo* [30]. The epithelial cells of the intestinal barrier have a high turnover rate, because they are quite sensitive against alterations of their endogenous environmental conditions involving a deficiency of the epithelium and immune/inflammation mediating cells [37].

As shown in this study, the cell metabolism of the IPEC-J2 cells was stimulated in a dose-dependent manner. However, a significant increase in cell metabolism was observed at the theoretically calculated Sanomucin concentrations of 250 µg/mL in blood fluid and 500 µg/mL in intestinal fluid. Since cell metabolism is the sum of all chemical reactions that keep cells alive and enable them to grow, divide, and respond to their environment, it is essential for maintaining cellular structure, repairing damage, and carrying out specialized functions.

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Especially the intestinal epithelium is a multicellular interface with intestinal epithelial cells as the inner layer, which absorb nutrients and form a physical and immune-mediated barrier against harmful components of the luminal environment. In addition, the intestinal epithelium is the most regenerative tissue in the human body and acts as a protective mechanism against injury and infection and homeostasis [38].

Cellular metabolic activity is directly linked with cell regeneration which is a fundamental biological process that enables organisms to replace damaged or dead cells and thus maintain homeostasis [39]. Promoting intestinal epithelial cell regeneration as demonstrated here for Sanomucin can result in an earlier restoration of the integrity and functionality of the affected mucous tissue area. In most regenerating processes, the closure of a defect in a given structure requires the production of new cells by proliferation and migration of cells [40]. As demonstrated here, the observed increase in metabolic and regenerative activity by Sanomucin might also act as a protection against tumor therapies often affecting the mucous tissues in the body [41].

Moreover, oxidative stressors such as tumor therapies can increase the production of reactive oxygen species in the body, overwhelming the antioxidant defense system and leading to oxidative damage of cell components such as DNA, proteins, and lipids. A common *in vitro* model to simulate exogenous oxidative stress is the use of hydrogen peroxide as a donor for reactive oxygen species acting on cultured cells [42-44]. This *in vitro* model was also used to examine whether Sanomucin might be able to reduce oxidative stress acting on intestinal epithelial cells. Although Sanomucin is not a high-dose antioxidant dietary supplement, it can prevent oxidative damage to healthy cells; Sanomucin is not expected to interfere with the treatment of cancer. The measurement data indicate that Sanomucin has marked potential to counteract oxidative stress and may help reduce the effects of oxidative stressors, thereby supporting the integrity and functionality of the intestinal barrier.

We also studied the effect of Sanomucin on the metabolism and endogenous superoxide anion generation of inflammation-mediating cells as the second cell type of this study. Here we observed that Sanomucin decreased the basal cell metabolism of functional neutrophils and, simultaneously, the dose-dependent decreased generation of superoxide anion radicals. *In vivo*, polymorphonuclear neutrophils are short-living granulocytes with a very high turnover rate and known as frontline effectors of the innate immune defense [45-48]. On the other hand, they are attracted by cytokines released into the blood in the course of an inflammatory process migrating into this tissue and generating superoxide anion radicals causing a further progression of the inflammation [49-52]. Thus, the observed decrease in radical generation after application of Sanomucin might also reduce an inflammatory process in the intestine *in vivo* caused by oxidative stress after tumor or other physically demanding therapies.

Limitations of the study

The present study is a preclinical study with cultured intestinal and inflammation-related cells. The test concentrations of Sanomucin were theoretically calculated from the recommended daily dosage and might be considerably lower after passage through the gastrointestinal tract and absorption in the small intestine. In addition, the preserving effect of Sanomucin has been also checked in a single test on intestinal epithelial layer integrity by measuring the transepithelial resistance of the layer which was increased by about 19 % by Sanomucin (data not shown).

CONCLUSIONS

The in vitro results presented here suggest that Sanomucin may support cellular processes relevant to mucosal resilience, including epithelial metabolic activity, regeneration following mechanical injury, resistance to oxidative stress, and the modulation of reactive oxygen species released by neutrophils. These mechanistic findings must be confirmed in controlled clinical trials before definitive conclusions regarding clinical efficacy can be drawn.

Conflict of interest

The present study was funded by Helixor Heilmittel GmbH, D-72348 Rosenfeld, Germany. The company made Sanomucin available for the experiments.

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Citation: Dartsch PC (2026) Effects of Sanomucin on Intestinal Epithelial Cells and Inflammation-Related Cellular Responses *in Vitro*. Appl Cell Biol, 14(2), [13-20]