Molecular basis of sodium butyrate-dependent proapoptotic activity in cancer cells

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Abstract

This review outlines the molecular events that accompany the antitumor action of sodium butyrate (NaBt). Butyrate, a low-molecular weight four-carbon chain volatile fatty acid (VFA) has been previously shown to withdraw cells from cell cycle or to promote cell differentiation, and finally to induce programmed cell death. Recent advances in molecular biology indicate, that this product of large bowel microbial fermentation of dietary fiber, might evoke the above-mentioned effects by indirect action on genes. NaBt was shown to inhibit histone deacetylase activity, allowing DNA binding of several transcription factors. Higher genomic activity leads to the higher expression of proapoptotic genes, higher level of their protein products and elevated sensitivity to death ligand-induced apoptosis. Cancer cells might be arrested in G1 phase of cell cycle in a p21-dependent manner. Proapoptotic activity of NaBt includes higher expression of membrane death receptors (DR4/5), higher level and activation of Smad3 protein in TGF-β-dependent apoptotic pathway, lower level of antiapoptotic proteins (cFLIP, XIAP) and activation of proapoptotic tBid protein. Thus, both intrinsic and extrinsic apoptotic pathways are stimulated to ampify the apoptotic signals. These effects are specific for tumor but not for regular cells. Unique properties of NaBt make this agent a promising metabolic inhibitor to retard tumorigenesis to suppress tumor growth.

Key words: sodium butyrate, apoptosis, cancer cells.

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Introduction

The "immune escape", also known as immunoediting, is evolutionary developed ability of cancer cells to avoid elimination by the immune system [1-3]. The main strategies used, such as ignorance, impaired antigen presentation, expression of immunosuppressive factors and molecules, tolerance induction and apoptosis resistance allow tumor cells to grow and develop [3]. The current efforts are focused on identification of the molecular mechanisms responsible for the inhibition of apoptotic signals and sensitization of cancer cells to natural cell death induction by metabolic inhibitors. Among various tested compounds also naturally derived substance i.e. sodium butyrate is a promising agent for future anticancer immunotherapy.

Sodium butyrate – multifunctional short-chain volatile fatty acid

Butyrate in a non-toxic short-chain fatty acid that is produced naturally during the microbial fermentation of dietary fiber in the colon [4]. Butyrate plays an important role in homeostasis of the colonic mucosa by inducing pathways of cell maturation, including cell cycle arrest, differentiation and apoptosis [5,6]. More interestingly, butyrate-mediated regulation of apoptotic pathways occurs also in colon cancer cells [7-9]. Noteworthy, the proapoptotic action of sodium butyrate (NaBt) is not limited to gastrointestinal tract but was also reported in chronic myelogenous [10] and myeloid leukemia [11], breast [12], prostate [13,14] and many other cancer types [15,16]. Despite of numerous studies demonstrating the antiproliferative effect of NaBt treatments, there is no universal explanation for this phenomenon. The review presents some of the postulated molecular mechanisms of sodium butyrate-mediated regulation of apoptosis process.

Sodium butyrate regulates gene expression by inhibiting histone deacetylase activity

Sodium butyrate and other short-chain fatty acids (SCFAs) are histone deacetylase (HDAC) inhibitors. The major biochemical change that occurs in cells treated with HDAC inhibitors is the hyperacetylation of histones [17]. Histone proteins package DNA into nucleosomes, and core histones can be acetylated on lysine residues of NH2-terminal tails. Acetylation and deacetylation are catalyzed by specific enzymes, histone acetyltransferese and HDAC, respectively [18]. Sodium butyrate causes histone hyperacetylation through a noncompetitive and reversible inhibition of HDAC [19]. Histone hyperacetylation neutralize the charge between histone tails and DNA, freeing this region of DNA for access to transcription factors and is generally associated with activation of specific genes [20,21]. NaBt is intensively tested in cancer research, keeping in mind, that chromatin modification is a key factor in the development of neoplasia (for example certain oncogenic transcription factors, such as leukemogenic transcription factor promote oncogenesis by deregulation of chromatin structure) [22].

Sodium butyrate regulates Sp1 transcription factor

The according to Kim et al. [7] one of the NaBt cellular targets is Sp1 transcription factor. NaBt treatment [2, 3 mM, 6 h] disrupted association of histone deacetylase with Sp1 in colon cancer HCT-116 and HT-29 cell lines. At the same time, the supershift analysis confirmed increased binding of Sp1 to DNA in sodium butyrate-treated nuclear cells extracts, which in turn led to chromatin decondensation and activation of DR5 gene transcription. As a consequence, the HCT-116 and HT-29 cells became refractory to TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) by up-regulated expression of DR5 protein, the TRAIL specific transmembrane receptor. Interestingly, NaBt was not able to activate the DR4 gene expression, which codes the second transmembrane TRAIL receptor. The selective expression of TRAIL receptors was also demonstrated in bladder [23] and breast cancer cells [12]. In colon cancer cells the NaBt-stimulated [0.5, 2 mM, 24 h] up-regulation of DR5 receptor allowed TRAIL to induce apoptosis process, which was visualized by procaspase-3 activation and PARP degradation. Simultaneously, the Western blot analysis showed the reduction of XIAP but not cIAP-1 and cIAP-2 antiapoptotic proteins level [7]. However, the authors did not prove that the expression of cIAPs is also Sp1-dependent.

Sodium butyrate induces G1 cell cycle arrest and sensitizes cancer cells to death ligands-induced apoptosis by p21-dependent pathway

The other target for NaBt action is p21. The NaBt-stimulated [5 mM, 24 h] histone hyperacetylation resulted in accumulation of acetylated histones H3 and H4 in colon cancer COLO-320 and SW1116 cell lines [24]. It is noteworthy, that the level of acetylated histone H3 and H4 at the domain containing the transcriptional start site in *p21/WAF-1* promoter and the binding sites of E2A transcription factor was significantly higher than that at another domain in promoter. Thus the authors observed the extended *p21* mRNA and p21 protein level [24]. p21/WAF-1 is the major suppressor of cyclins (A-H) and their associated cyclin-dependent kinases (cdk) [25]. The balance between the activation and inhibition of cyclin/cdk activities determines whether or not a given cell will proceed through the cell cycle and, as such, may contribute to the development of neoplasia. Archer et al. [26] demonstrated that NaBt administration [5 mM, 1-48 h] retarded HT-29 colon cancer cells growth and concomitantly decreased cyclin B1 (cB1) protein level. Further studies revealed that the delayed reduction in cB1, beginning at 6 h with maximal changes at 24 h (90%), contrasted with the early induction of p21 mRNA at 2 h. To verify the relationship between cB1 and p21 proteins, the HCT-116 p21 wild-type (+/+), heterozygote (+/-) and mutant (-/-) cells were used. The Northern-blot analysis showed that NaBt treatment caused dramatic decrease of cB1 expression in +/+ and +/-, but not in -/- cells. These results proved that p21 plays a critical role in butyrate-mediated repression of cB1 in colon cancer cells. This repression occurs through cis-element within 90-bp of the *cB1* gene transcriptional start site. The authors hypothesized, that histone hyperacetylation allows direct binding of p21 to DNA through protein-protein interactions. Although p21 is known to contain a zinc finger motif, generally seen in transcription factors, it has not been shown to play a role of transcription factor. It is possible that the p21-DNA interaction is mediated by cdk proteins, which could be bound to the amino-terminal portion of p21 [27]. Interestingly, Archer et al. [26] reported that in 90-bp region upstream of the *cB1* transcriptional start site, several consensus sequences for various transcription factors are localized, such as: heat shock factor (HSF), NF-Y and Sp1. Thus the transcription regulation of gene expression could be more complex and hard to predict.

On the other hand, Wang et al. [16] claimed that in primary effusion lymphoma (PEL), a peculiar type of B cell non-Hodgkin lymphoma cells, co-infected with Epstein-Barr virus (BCBL-1 cells) NaBt regulates cell cycle-related proteins and cause the growth inhibition but in a p21/WAF-1-independent manner. The Western blot analysis revealed the decreased cyclin-dependent kinase (cdk) 2, cdk4 and cyclin A proteins level in NaBt-treated cells, but at the same time there were no changes observed in p21/WAF-1 expression. The authors hypothesized that distinct results could be explained by the presence of virus, which could modulate cell response [16].

The p21/WAF-1 action was also examined by VanOosten et al. [13] in three prostate cancer cell lines: ALVA-31, DU-145 and LNCaP and by Earel et al. [23] in bladder tumor cells. Similarly to Archer et al. [26], NaBt [5 mM, 24 h] stimulated p21/WAF-1 activity, that in turn increased the percentage of cells in G1 cell cycle phase [13,23]. Additionally, in prostate cancer cells NaBt increased the responsiveness to TRAIL-induced cell death, further confirmed by the flow cytometry analysis [13]. Moreover, the quantitative real-time PCR revealed a modest up-regulation

in DR5 (TRAIL-R2) mRNA level after NaBt treatment, but no changes in DR5 death receptor protein level on cell surface was detected. These observations are contradictory to the previously described results published by Kim et al. [7], Earel et al. [23] and Chopin et al. [12] who found significantly higher level of DR5 surface protein after NaBt administration. VanOosten et al. [13] concluded that in prostate cancer cells additional molecular mechanism exists, which supports TRAIL-induced apoptosis. Based on Izeradjene et al. [28,29] and Ravi et al. [30] studies, the role of casein kinase II (PKCK2), which is also engaged in TRAIL resistance, was evaluated. The mechanism of the change in phenotype was found to lie in the connection between PKCK2 and caspase-2 [31]. When PKCK2 is down-regulated, procaspase-2 is dephosphorylated, allowing it to dimerize and become activated. The activated caspase-2 then processes procaspase-8 monomers between the large and small subunit, so that procaspse-8 can be fully activated by cleavage whenever TRAIL is recruited to DR4/5 death initiating signaling complex (DISC) after TRAIL-DR4/5 ligation. Interestingly, for the first time VanOosten et al. [13] demonstrated that PKCK2 activity is regulated by HDAC inhibitors, such as NaBt. The NaBt administration resulted in highly significant inhibition of PKCK2 activity, accompanied by the increased caspase-2 activity. Moreover, the immunoprecipitation analysis confirmed the elevated level of cleaved p43/41 procaspase-8 fragment in DR5-DISC complex. According to previously described scenario, the addition of the specific procaspase-2 inhibitor, Z-VDVAD-fmk totally abrogated NaBt-induced increase in TRAIL sensitivity. Finally, if cells were treated with 4,5,6,7--tetrabromobenzotriazole (TBB) prior to TRAIL treatment, the PKCK2 inhibitor caused a number of cells to undergo apoptosis, when compared to either agent used individually. Summing up, these results demonstrated that NaBt can sensitize tumor cells to TRAIL-mediated apoptosis by inhibiting PKCK2 activity, which in turn leads to caspase-2 activation and the processing of procasapse-8 into active form when tha latter is recruited to the DR-DISC complex. Within the context of apoptotic signal transduction pathways, the location of caspase-2 in this pathway has been historically lacking. Nevertheless, the activation of caspase-2 in primary effusion lymphoma after NaBt administration [3 mM, 18 h] was previously reported by Wang et al. [16]. the authors did not explain the mechanism of NaBt-induced p48 procaspase-2 to p33 cleavage. They supposed that the observed increase in active caspase-2 protein level resulted from oxidative stress [16]. However, the use of various antioxidants, such as vitamin C or catalase, did not protect tested cells from NaBt-induced apoptosis. Therefore, the above-mentioned observations reported by VanOosten et al. [13] shade more light on the molecular mechanism of NaBt-mediated sensitization of cancer cells to TRAIL-induced cell death.

Sodium butyrate sensitization of cancer cells to death ligands-induced apoptosis is mediated by down-regulation of antiapoptotic proteins

In accordance to former chapter, the NaBt is able to sensitize various cancer cells to TRAIL-induced cell death [7,13,23]. The identification of cellular targets for NaBt led these authors to draw similar conclusions pointing to the NaBt-dependent molecular mechanism of restored susceptibility of tumor cells TRAIL. Hitherto, no detailed study was done concerning the co-operation of NaBt with other death ligands (TNF-α, FasL). However, some reports suggest the presence of other cellular targets for sodium butyrate. NaBt up-regulates signals of death ligand-induced apoptosis. According to Chopin et al. [12] NaBt [1 mM, 6-48 h] modulates the TNF-R1, TNF-R2, Fas-R/CD95 death receptors in MCF-7 breast cancer cell line. At the same time, in NaBt-treated cells the Western blot analysis clearly showed the elevated level of FADD protein, one of the DISC complex components. The stimulated expression of transmembrane receptors and DISC components resulted in extensive cell death after TNF-α or FasL exposure [0.1 ng/mL, 18 h], what was visualized by Hoechst staining and confirmed by caspase-8 activation. Similar observations in colon cancer cells were previously demonstrated by Giardina et al. [32] and Hara et al. [33]. Additionally, Chopin et al. [12] indicated that mitochondria are involved in NaBt-induced apoptosis. TNF-α or FasL and NaBt co-treatments increased the level of tBid (truncated Bid), which is able to translocate to mitochondrial membrane and to induce release of cytochrome c from mitochondria to cytosol. Cytosolic cytochrome c favors the activation of caspase-9, which in turn activates downstream caspases [34]. In MCF-7 breast cancer cells the release of cytochrome c and caspase-9 activation were inhibited in the presence of Z-LETD-fmk, caspase-8 inhibitor. It was concluded that mitochondria-dependent apoptotic pathway is activated as a consequence of ligand-receptor complexes formation. It is not clear, whether NaBt modulates the mitochondrial apoptotic pathway, or just initiates execution of programmed cell death (PCD).

The second possible scenario of sensitization of various cancer cells to death ligands-induced cell death is the elimination of antiapoptotic proteins, which are able to inhibit trunsduction of death signal in the cell interior. One of such proteins is cFLIP (FLICE-inhibitory protein) protein bound to DISC complex of TNF-R1, DR4 and -5, and Fas-R [35]. By direct interaction with FADD protein, cFLIP diminishes or totally blocks death signals by competitive inhibition of caspase-8 activation [35]. A critical role of cFLIP in the resistance of certain cancers to death ligand-induced cell death was demonstrated. Upon treatment with certain cytokines increased sensitivity to cell death of cancers cells was associated with apparent reduction in cellular levels of cFLIP [36,37]. According to Hernandez et al. [38] NaBt could be considered as another potent agent that could serve as a useful adjunct for the treatment of metastatic colorectal cancer. They found that NaBt treatment [5 mM, 24 or 48 h] inhibits cFLIP expression in three human colon cancer cell lines: KM12C, KML4A and KM 20. Moreover, when cells were co-treated with NaBt [5 mM] and TRAIL [100 ng/mL], both the caspase-3 assay and Annexin-V immunofluorescenct assay showed apoptosis induction. The similar results were obtained by Natoni et al. [39], who observed the significant reduction of cFLIP level after NaBt administration in pancreatic cancer cells. As a result, cells became responsive to FasL-induced programmed cell death. Noteworthy, at the same

time the intrinsic apoptotic pathway was activated, which is in accordance to data previously reported by Kim et al. [7] in MCF-7 breast cancer cell line. The same authors also noticed NaBt-mediated elimination of the antiapoptotic proteins, such as XIAP. It was supposed, that NaBt could efficiently regulate the presence of various antiapoptotic proteins in cancer cells, supporting the objectives for immunotherapy.

Sodium butyrate up-regulates TGF-β signaling pathway in cancer cells

Transforming growth factor beta (TGF-β) is expressed, for instance in gut epithelium and serves an important role in negative regulation of the proliferation of enterocytes and colonocytes. TGF-β is also a potent tumor suppressor by inhibiting cellular proliferation and inducing apoptosis [40, 41]. However, most cancer cells are resistant to the TGF-β-induced apoptosis by acquiring defects of various components of TGF-β signaling pathway. For example, TGF-β I receptor [42], type II receptor [43], Smad2 [44] and Smad4 [45] have been shown to be either mutated or down-regulated in human colorectal cancers. TGF-β mediate signals through its binding to a cell surface receptor complex, which subsequently phosphorylates Smad2 and Smad3. The phosphorylated Smad2 or Smad3 form a heteromeric complex with Smad4, which translocates into nucleus and regulates transcription of target genes [46]. Nguyen et al. [8] described for the first time, the sodium butyrate-mediated regulation of TGF-β pathway. In HT-29, KM12C, KM12L4A, and KM20 colon cancer cell lines NaBt significantly induced the Smad3 protein expression, what was visualized by Western blot analysis. Moreover, NaBt up-regulated Smad3 activation by its extensive phosphorylation, which allowed Smad3 to translocate to the nucleus. The quantitive RT-PCR analysis revealed that the consequences of increased Smad3 activation were the higher plasminogen activator inhibitor-1 (PAI-1) and cyclooxygenease-2 (COX-2) mRNA levels, gene products engaged in proces of carcinogenesis. Since NaBt enhances TGF-β signaling and TGF-β is an important tumor suppressor the authors next examined whether NaBt enhances the tumor suppressor function of TGF-β. The tumor suppressor function was attributed to its ability to inhibit cell cycle progression and induce apoptosis. Although, the authors did not identify the molecular mechanism, it was found that NaBt and TGF-β synergistically inhibit anchorage-independent growth of colon cancer cells. The described data revealed a novel mechanism that may explain in part the beneficial effects of sodium butyrate in decreasing risk of colon cancer.

Sodium butyrate specifically affects malignant cells

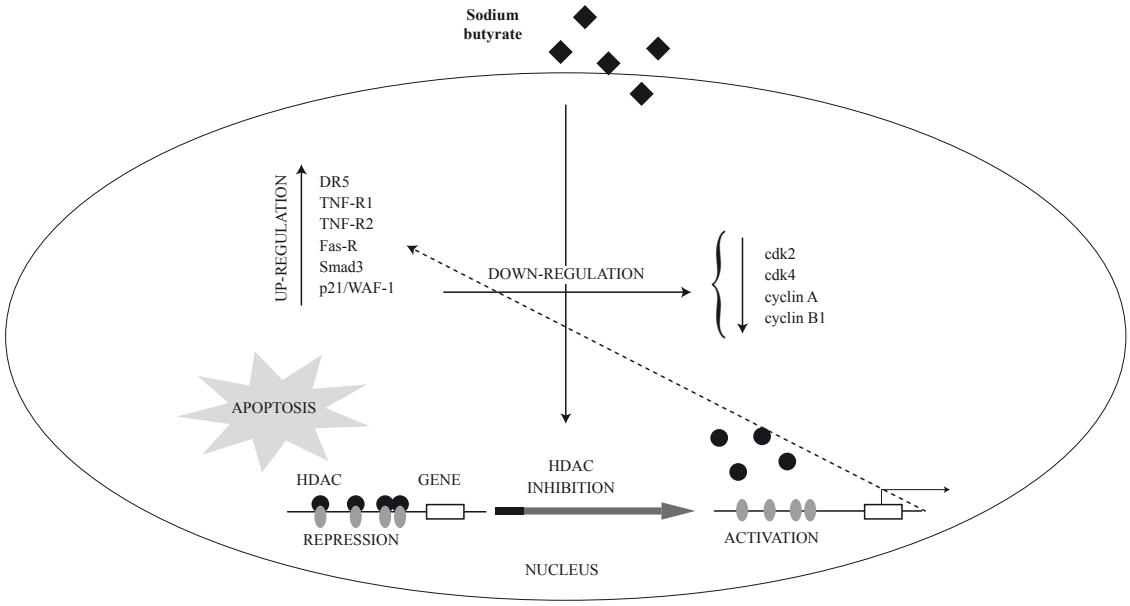
Studies of intensive immunotherapy revealed several metabolic inhibitors, such as cycloheximide [37], actinomycin D [47], anisomycin, harringtonine [48] and other metabolic inhibitors, which are able to modulate the resistance of various cancer cells to cytokine-induced cell death. However, the clinical use of several tumor cell death promoting agents is limited, because they act non-specifically and are often cytotoxic. Thus, non-toxic NaBt seems to be the ideal agent in future anticancer immunotherapy. Earel et al. [23] showed that the NaBt-mediated sensitization to TRAIL-induced PCD is specific ultimately for cancer cells, whereas do not affect normal cells in bladder. In normal bladder epithelial cells (SVHUC-1) co-treatment with NaBt [5 mM] and TRAIL [10-1000 ng/mL] caused only 20% of cells to die, and barely if TRAIL was used in the highest concentration. To understand why the SVHUC-1 cells were not sensitized by NaBt to TRAIL, the TRAIL-R1 (DR4) and TRAIL-R2 (DR5) expression levels were assessed after NaBt administration. In both cases the expression of transmembrane TRAIL receptors was unchanged. These observations are in contrast to those described previously for bladder cancer represented by T24 cell line, They responded with increased DR5 expression after NaBt treatment. Thus, the authors concluded that NaBt may be a viable alternative treatment.

Other interesting studies were described by Liu et al. [49], who evaluated the affect of sodium butyrate on 1,2-dimethylhydrazine (DMH)-induced colon tumorigenesis in mice. The mice of Kunming species were divided into five groups and received relevant treatments: control group (saline), DMH alone group (with subcutaneous injection of 30 mg/kg of DMH weekly for eleven weeks), DMH plus low dose of NaBt group (1.25 mol/kg, 24-week coloclysis), DMH plus high dose of NaBt group (2.5 mol/kg, 24-week coloclysis) and high dose of NaBt group. The mice were killed in batches at the 12th, 18th and 24th weeks of carcinoma induction separately. The incidence of colorectal tumor in each group was evaluated. Meanwhile, the general condition, body weight, liver and renal functions and pathological changes of liver, kidney, lungs and pancreas of the mice were also measured. The obtained results revealed that at the 24th week of study the tumor incidence was 95% in DMH mice group, 45% in DMH plus low dose of NaBt group, and 15% in DMH plus high dose of NaBt group. More importantly, no tumors were observed in control group and high dose of NaBt group. No differences in general condition, body weight and liver and renal functions of mice were observed between control and high dose of NaBt group ($P>0.05$). Furthermore, no pathological changes in lungs, livers and kidneys were observed in the mice with high dose of NaBt group. The described results confirmed that NaBt is nontotoxic for normal cells, moreover, they suggest that NaBt could protect against experimentally-induced colon carcinogenesis.

Summary

Herein, the various molecular mechanisms of proapoptotic sodium butyrate action in cancer cells are described. On transcription level, NaBt affects histone deacetylase activity. The up- or down-regulation of specific genes results in the antiapoptotic proteins elimination, such as cFLIP, XIAP and/or extensive synthesis of transmembrane receptors or components of various apoptotic signaling pathways (TGF-β,TRAIL) (*Fig. 1*). All these cellular changes help to restore the natural processes of cancer cell deletion. Additionally, the presented *in vitro* and

Figure 1. **Tentative model for the mechanism of sodium butyrate-mediated apoptosis induction. Butyrate-dependent inhibition of histone deacetylase (HDAC) activity results in the up-regulation of DR5, TNF-R1, TNF-R2, Fas-R, Smad3 and p21/WAF-1 gene transcription. As a consequence of p21/WAF-1 activation the cdk2, cdk4, cyclin A and cyclin B1 are down-regulated. Presented changes in gene transcription facilitate apoptosis induction in cancer cells [based on Kim et al., Carcinogenesis 2004; 25(10): 1813-20]**



in vivo studies showed that sodium butyrate specifically affects malignant cells but not the normal ones. Thus, sodium butyrate should be seriously considered as an anticancer treatment or an adjuvant in novel immunotherapy.

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