



Fiber-derived butyrate and the prevention of colon cancer

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Inhibition of the enzyme histone deacetylase by butyrate results in the direct transcriptional upregulation of the cyclin-dependent kinase inhibitor p21/Cip1/WAF1. We discuss a small-molecule-mediated signaling pathway to explain the suspected anti-colon-cancer properties of fiber-derived butyrate.

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Fiber, butyrate and colon cancer

Studies relating diet to oncogenesis suggest a correlation between fiber intake and the occurrence of colorectal cancer (for reviews see [1]). Experiments focusing on the role of dietary fiber in colonic homeostasis indicate the importance of fiber-derived fermentation products produced by anaerobic microflora within the lumen of the large intestine [1]. In particular, the microbial production of a short-chain fatty acid, butyric acid, appears to be important for proper epithelial cell regulation [1]. Unfortunately, attempts to determine precisely the connection between fiber, butyrate and colon cancer have been complicated by the experimental challenges inherent in studying whole organisms and the inability to reproduce *in vivo* conditions accurately in tissue culture. Butyrate serves as a primary energy source for colonocytes [1], and numerous studies on human biopsies and animal subjects indicate that butyrate has a proliferative effect on normal colonic epithelium (reviewed in [1]). Nevertheless, butyrate has anti-proliferative and differentiation-inducing effects on various human colon carcinoma cell lines, as well as on other normal and neoplastic cells, at the physiological (millimolar) concentrations obtained *in vivo* [1–3]. Despite the apparent discrepancy between *in vivo* and *in vitro* studies, the anti-growth activity of butyrate on tissue-culture cells has led to the hypothesis that butyrate protects against colon cancer by promoting differentiation, cell-cycle arrest and apoptosis of transformed colonocytes [1].

The treatment of mammalian cells *in vitro* with low millimolar concentrations of butyrate causes pleiotropic effects on cellular physiology, including changes in the cell membrane, the cytoskeleton, the cell cycle and gene transcription [4]. At least part of the effects induced by butyrate can be attributed to the direct inhibition of the enzyme histone

deacetylase (HDAC) [5]. Reversible acetylation of the ϵ -amino groups of lysine residues on the amino-terminal domains of histones is important for nucleosome assembly, chromatin structure and transcriptional regulation (for reviews see [6–8]). Although the precise mechanism(s) and downstream effects of histone acetylation are poorly understood, a hallmark of HDAC inhibition is cell-cycle arrest and the induction of cellular differentiation (reviewed in [9]).

The possibility that butyrate-mediated inhibition of HDAC is, in part, responsible for the effects seen on cells *in vitro* is supported by studies using other HDAC inhibitors that mimic the cytological effects of butyrate. The inhibitors, which are microbial metabolites, were initially identified by their ability to revert oncogenic tumor cells to a normal, detransformed morphology (reviewed in [9]). The insightful observation that one of these detransforming agents, trichostatin A (TSA), was in fact a highly potent (nanomolar K_i) inhibitor of an HDAC activity *in vitro* and an inducer of histone hyperacetylation *in vivo* paved the way for the discovery of a variety of natural products with HDAC-inhibitory properties (Figure 1) [9–12]. These findings also led to the purification and cloning of HDAC1 using the HDAC inhibitor trapoxin B (TPX) as a molecular probe [13]. These and subsequent studies using HDAC inhibitors indicated a role for HDAC in control of the G1/S and G2/M transitions of the cell cycle, as well as in cellular differentiation and senescence [9,14].

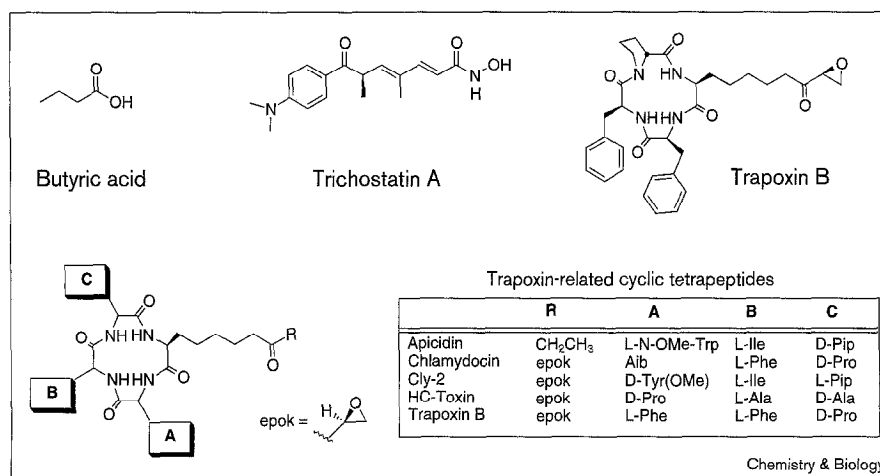
The recent demonstration that histone acetyltransferases (HATs) and HDACs are transcriptional regulators [6,8,15,16] provides evidence to support the argument that cytological changes induced by HDAC inhibitors are the downstream effects of altered transcription. In general, HDAC inhibitors specifically upregulate or downregulate the transcription of genes linked to cell proliferation and differentiation, such as *myc* [6,17–19], *fos* [17], and the gene encoding gelsolin [9], rather than altering the transcription of housekeeping or constitutive genes, such as the gene encoding GAPDH (see [6] and references therein). The effects of deacetylase inhibitors on cellular processes vary depending on the cell type and the micro-environment of the cell. In the colon, butyrate has the capacity to act as a signaling molecule, being able to traverse the cell membrane of colonocytes, bind to its cognate receptor HDAC, and mediate effects on gene transcription, resulting in dynamic changes in cell physiology.

Induction of p21 by histone deacetylase inhibitors

Among the genes that are directly transcriptionally upregulated in cells treated with butyrate or other HDAC

Figure 1

Histone deacetylase inhibitors. Several natural products have recently been shown to inhibit the enzymatic activity of HDAC. The small organic molecules vary dramatically in their chemical structure. The simplest compound, butyric acid, is a short-chain fatty acid with an HDAC inhibitory K_i in the low millimolar range (~2 mM). Trichostatin A (TSA) and trapoxin B (TPX) were identified as fungal antibiotics with potent differentiation-inducing properties [9]. TSA, TPX and trapoxin-related compounds inhibit HDAC potently at low nanomolar concentrations. TSA is a hydroxamic acid that is not structurally related to the family of trapoxin-related compounds. Trapoxin-related compounds are cyclic tetrapeptides containing an extended aliphatic sidechain that is required for biological activity and usually contains an epoxyketone (epok) moiety. Apparently each of these compounds possesses a lysine sidechain mimic that potentially interacts with the catalytic elements of the enzyme. Aib, α -aminoisobutyric acid; Pip, pipercolic acid.



inhibitors is the cyclin-dependent kinase (CDK) inhibitor p21/Cip1/WAF1 [20,21]. Cyclin-CDK complexes phosphorylate key proteins involved in cell-cycle checkpoints and the DNA-replication machinery, thereby facilitating cell-cycle progression. The regulated formation of active cyclin-CDK complexes is governed principally by CDK inhibitors (CKIs; for review see [22]). The CKI p21 binds and inhibits a range of cyclin-CDK enzyme complexes in addition to directly associating with proliferating cell nuclear antigen (PCNA) and inhibiting PCNA-dependent DNA replication *in vitro* [22,23]. Transcription of p21 is controlled by multiple factors including a p53-dependent mechanism induced by DNA damage [22,24] and a TGF β -mediated mechanism [25]. In addition, p21 message levels are elevated by numerous differentiating agents including phorbol ester, dimethylsulfoxide, all *trans*-retinoic acid and vitamin D (1,25-dihydroxyvitamin D₃), implicating p21 in cellular differentiation [8,21,25-27].

The effect of butyrate and other HDAC inhibitors on cells is closely paralleled by the effects of p21 expression. Studies indicate a role for p21 in the regulation of the G1 [22], S [28,29] and G2 [22,30,31] phases of the cell cycle. Furthermore, the formation of polyploid nuclei in hepatocytes overexpressing p21 [31] is reminiscent of the butyrate-induced polyploidism observed in certain mammalian cell lines *in vitro* [32]. Finally, senescent cells demonstrate a failure to downregulate p21 mRNA, indicating a role for p21 in establishing and/or maintaining senescence [33]. Given the prominent role of p21 in cell-cycle control and cellular differentiation, and the phenotypic similarities between p21 induction and HDAC inhibition, it is possible that direct upregulation of p21 is primarily

responsible for the cytological effects of HDAC inhibitors. The abundance of naturally occurring HDAC inhibitors and their ability to disrupt the transcription of cell-cycle-specific genes suggest a convergence of microbial evolution towards a common cellular target, HDAC, and emphasize the importance of this enzyme in the cell cycle and cellular differentiation. Potentially, combinatorial chemistry could be used to identify novel HDAC inhibitors for use in the study of histone acetylation and for use as possible drugs in the treatment of certain cancers.

Direct regulation of p21 transcription by histone deacetylase

Butyrate rapidly induces p21 mRNA within 3 hours of treatment, even in the presence of protein-synthesis inhibitors [20]. These results, combined with the demonstration that TPX induces p21 [34], strongly argue that p21 transcription is directly repressed by HDAC. Although the mechanism(s) by which histone acetylation affects transcription remains unresolved, experiments *in vitro* suggest that transcription factors gain increased accessibility to their DNA-binding sites on acetylated templates [6,15]. In the case of the p21 promoter, this implies that transcription factors capable of activating p21 transcription are present in the nucleus but have a reduced affinity for their DNA-binding sites when the local chromatin environment is deacetylated. Treatment with HDAC inhibitors shifts the equilibrium in the direction of acetylation, resulting in the binding of transcriptional activators and the subsequent production of p21 mRNA.

HDACs might regulate transcription either by globally deacetylating histones or by targeting deacetylation to

specific gene regulatory elements. In either case, only genes with regulatory elements sensitive to acetylation will be affected. Evidence for both mechanisms exists and the two models need not be mutually exclusive. On the one hand, HDAC inhibitors cause a detectable increase in total cellular histone acetylation states, reaching significant levels in approximately 8 hours of drug treatment [35], which seems to indicate that a large percentage of nucleosomal histones are normally deacetylated by a mechanism that does not require DNA replication or *de novo* nucleosome assembly. The quantity of cellular histones becoming hyperacetylated argues for a global non-targeted mechanism. On the other hand, recent experiments demonstrate a physical link between HDACs and other known transcriptional regulators and co-repressors that either bind DNA directly or are associated with DNA-binding proteins (for review see [6]). HDAC2 (mRPD3) was identified by Seto and colleagues [36] as a YY1-interacting protein. YY1 (Yin Yang 1) is a zinc-finger-containing DNA-binding transcription factor that represses or activates transcription depending on promoter context [37]. Virtually all genes regulated by YY1 are associated with cell growth, differentiation or development [37]. Multiple mechanisms for YY1-regulated transcription have been proposed, including those that rely on the ability of YY1 to associate with coactivator and corepressor proteins (for review see [37]). Overexpression of HDAC2 enhances YY1-mediated repression of a reporter gene containing YY1-binding sites and HDAC2 is capable of autonomous repression when fused to a GAL4 DNA-binding domain [36]. These findings suggest that HDAC might be targeted to specific promoter or enhancer regions by YY1 to regulate the transcriptional activity of genes through localized histone deacetylation.

A potential YY1-binding site in the p21 promoter

Given that HDAC inhibitors upregulate p21 transcription directly, it is possible for HDACs to be targeted to p21 promoter elements by specific HDAC-associated DNA-binding transcription factors, such as YY1. Interestingly, we have identified a putative YY1-binding site in the promoter of the p21 gene (Figure 2a). A combination of *in vivo* and *in vitro* experiments have elucidated the binding-site selection characteristics of YY1 [37]. A high affinity YY1-binding core motif, ACAT, is recognized by YY1 when this site is surrounded by flanking nucleotides showing a certain degree of variability [37]. The nucleotide sequence, 5'-GGACATTGA-3', on the anti-sense strand of the p21 promoter at position -767 to -759, complies with the requirements of a YY1-binding site.

The p21 gene is also regulated by the hormonal form of vitamin D, resulting in terminal differentiation of myeloid leukemia U937 cells [27]. The vitamin D-responsive element (VDRE) is composed of two direct repeats separated by a three nucleotide spacer (DR-3). The proposed YY1-binding site overlaps with the 3' end of the downstream

VDRE direct repeat (Figure 2a). The liganded vitamin D receptor (VDR) binds to the downstream direct repeat in association with its heterodimeric partner, retinoid-X-receptor (RXR), which occupies the upstream direct repeat [27]. It is possible that YY1 represses the p21 promoter by replacing VDR on the downstream half-element of the VDRE in the absence of vitamin D. Experimental evidence for a YY1 DNA-binding displacement model exists for numerous promoters [37]. In particular, displacement of VDR by YY1 has been demonstrated in the case of the osteocalcin gene, a bone-specific calcium-binding protein expressed only in differentiated osteoblasts [38]. The similarities between the p21 gene and the osteocalcin gene VDRE/YY1 elements are striking. Both are DR-3 elements, as expected for vitamin-D-responsive genes, and the position of the putative YY1-binding site in the p21 promoter overlaps the 3' portion of the downstream direct repeat, mimicking the position of the established YY1-binding site in the osteocalcin promoter (Figure 2b). The principal difference between the two VDRE/YY1 sites is that the YY1 site in the p21 promoter is in the opposite orientation to that seen in the osteocalcin gene. Experiments demonstrating that YY1 can bind to the p21 promoter and demonstrating the ability of YY1 to repress p21 transcription in an HDAC inhibitor-dependent fashion can be performed to support a functional role for YY1 in p21 transcription.

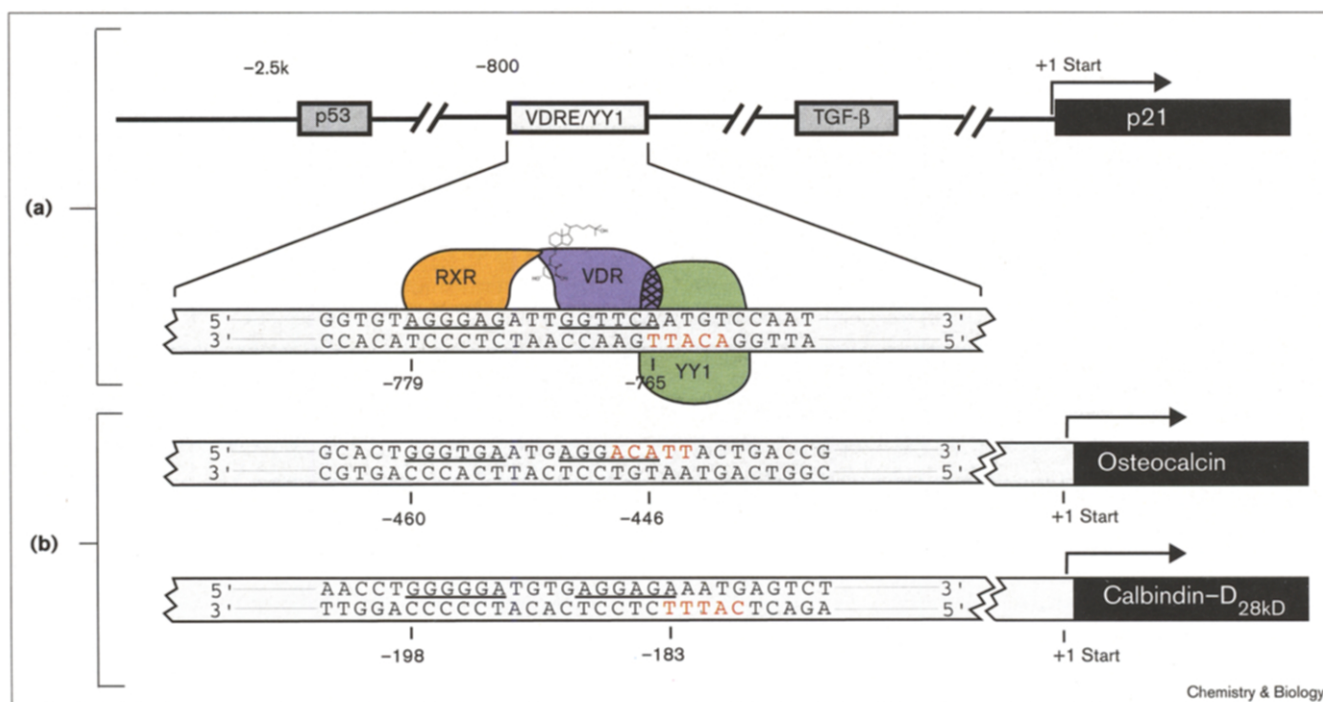
Analysis of another vitamin-D-responsive gene, calbindin-D_{28kD}, reveals the presence of a partially conserved YY1-binding site in this promoter as well (Figure 2b). In a similar fashion to the p21 and osteocalcin promoters, the putative YY1 site, 5'-ACTCATTTC-3' partially overlaps the downstream half-site of the reported VDRE (Figure 2b). Notably, calbindin-D_{28kD} is induced by butyrate, and the butyrate-response element (BRE) has been mapped to a region directly downstream of the proposed YY1-binding site [39]. Curiously, a transiently transfected construct containing the BRE but lacking the YY1 site retains butyrate inducibility following prolonged drug treatment [39], suggesting that the BRE is still deacetylated in the absence of the VDRE/YY1 element. Related BREs have been found in several butyrate-sensitive and HDAC-inhibitor-sensitive promoters [39,40] (Figure 2b). No regions of the p21 promoter show significant homology to the calbindin-D_{28kD} BRE, although transient transfection experiments implicate two Sp1-binding sites near the TATA box (nucleotides -101 to -77) as being important for activation by butyrate [20]. These mapping experiments suggest that a promoter lacking the putative YY1-binding site retains sensitivity to butyrate. Unlike the native p21 gene, however, which is strongly induced by butyrate within 3 hours, recombinant reporter genes containing truncated forms of the p21 promoter require significantly longer time periods of butyrate treatment before showing detectable levels of induction [20]. The discrepancy in the time courses may reflect the potential problems associated with studying chromatin

regulation in artificial transcription systems. It will be necessary to study the native p21 promoter in its natural chromatin context to ascertain the involvement of p21 regulatory elements in maintaining particular acetylation states.

The conserved architecture of the native p21, osteocalcin and calbindin- D_{28kD} VDRE/YY1 promoter elements suggests the potential for a related mechanism of transcriptional regulation in which certain vitamin-D-responsive genes recruit YY1 and an associated HDAC to enhance transcriptional silencing. The system described here provides two independent signaling mechanisms for altering transcription of the p21 gene. In one case 1,25-dihydroxyvitamin D_3 binds to the VDR, thereby facilitating the binding of VDR to DNA and the subsequent transcriptional activation of p21. In the other case, butyrate binds to a targeted HDAC and inhibits its transcriptional repression function, leading to p21

transcription. The similarities of these small molecule-dependent signaling pathways are shown in Figure 3. We have compiled the available data and note an intriguing correlation between YY1-regulated genes and sensitivity to HDAC inhibitors (Table 1). Genes repressed by YY1 are activated by deacetylase inhibitors, whereas genes activated by YY1, such as *cyclin D1*, are downregulated by HDAC inhibitors. This is consistent with both a positive and a negative role for HDACs in transcription (for review see [6,9,15]). Not all promoters affected by deacetylase inhibitors contain both a YY1-binding site and a VDRE, however, and further experiments are warranted in order to determine the generality of these observations and the precise role for each component involved. More recent advances in transcript-array technology might aid in the identification of genes transcriptionally altered by HDAC inhibitors and might also provide a method of determining potential differences in

Figure 2



(a) A putative YY1-binding site in the cyclin-dependent kinase inhibitor p21 promoter. Several transcriptional regulatory elements have been identified in the promoter/enhancer regions upstream of the p21 transcription start site. These elements include a DNA-damage-responsive p53-binding site [22,24] and a transforming growth factor β (TGF- β) responsive element [25]. The p21 promoter also contains a DR-3 type (direct repeat spaced by 3 nucleotides) vitamin D-responsive element (VDRE) [27] which is underlined. In the presence of vitamin D (1,25 dihydroxy vitamin D_3), a liganded vitamin D receptor (VDR) heterodimerizes with the retinoid X receptor (RXR). The VDR-RXR heterodimer binds to the VDRE as indicated. A putative ACAT-motif YY1-binding site is highlighted in red on the antisense strand. This site consists of a core CAT YY1-binding sequence flanked by a variable consensus sequence. The YY1 site overlaps the downstream direct

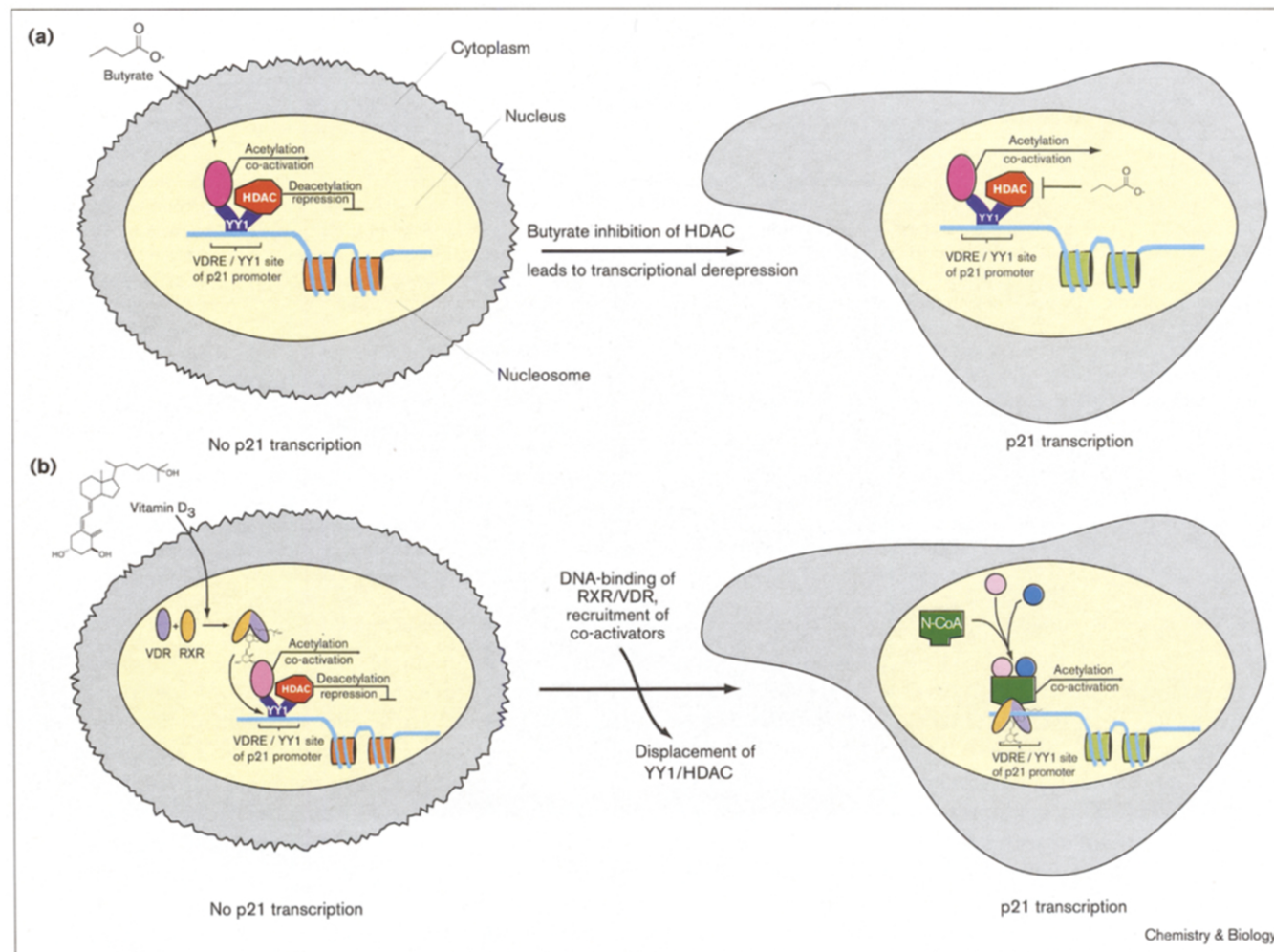
repeat of the VDRE, possibly allowing competitive binding between YY1 and RXR/VDR for the DNA-regulatory site. (b) Alignment of the osteocalcin and calbindin- D_{28kD} promoters with the p21 promoter. Transcription of both the osteocalcin and calbindin- D_{28kD} genes is induced by vitamin D and their VDRE elements are shown underlined. Both genes also contain a consensus YY1-binding site (putative in the case of calbindin- D_{28kD}) shown in red overlapping the downstream direct repeat VDR-binding site either on the sense strand (osteocalcin) or the antisense strand (calbindin- D_{28kD}). YY1 has been shown to compete with VDR/RXR heterodimers for binding to the osteocalcin promoter VDRE [38]. Unlike the p21 and osteocalcin promoters, both of which contain DR-3 VDREs, calbindin- D_{28kD} contains a DR-4 site [39]. The effects of these differences in the DNA-binding sites and in orientation of the YY1-binding site on transcription are unknown.

HDAC-inhibitor specificities. For example, the HDAC family members may regulate different genes allowing for the possibility of inhibitors that affect only a subset of HDAC-regulated genes, similar to the differential effects mediated by steroid hormones in transcription.

Interpretation of data derived from HDAC inhibitor studies is complicated by the possibility of indirect transcriptional

effects caused by hyperacetylation. Furthermore, not all genes directly influenced by HDACs will require YY1, nor is YY1 necessarily acting alone in recruiting HDACs. HDACs are thought to associate with the nuclear co-repressor, N-CoR, and the related SMRT (silencing mediator of retinoid and thyroid hormone receptors) proteins, as well as with the mSin3 co-repressor, to form a multi-protein transcriptional regulatory complex. Evidence suggests that

Figure 3



A model for transcriptional regulation of the cyclin-dependent kinase inhibitor p21/Cip1/WAF1 by a VDRE/YY1 regulatory element in the p21 promoter. Two independent small molecule-mediated signaling pathways may affect the transcriptional response of the p21 gene in colonocytes. A schematic representation of a colon cell is shown (cytoplasm in gray and nucleus in yellow). Both butyrate (a) and the hormonal form of vitamin D (b) can traverse the plasma and nuclear membranes and bind their intracellular receptors causing changes in transcription that result in cell-cycle arrest and differentiation (represented as a change in cellular morphology). Vitamin D binds the vitamin D receptor (VDR; purple) and butyrate binds HDAC (red). A putative YY1-binding site in the p21 promoter is occupied by YY1 under non-inducing conditions (DNA is shown in blue). YY1 represses transcription, in part through association with the co-repressor HDAC. HDAC may mediate localized deacetylation of nucleosomal histones,

shown here as red cylinders when deacetylated and green cylinders when acetylated. Derepression of p21 transcription can occur in two ways, either (a) through the inhibition of HDAC by butyrate, a short-chain fatty acid produced by colonic bacteria during the fermentation of certain forms of dietary fiber, or (b) through VDR-mediated activation via the ligand-dependent association of VDR with its heterodimeric DNA-binding partner RXR (retinoid X receptor; orange) which binds to the VDRE and subsequently recruits a transcriptional coactivator complex. This complex may include the coactivator/HAT N-CoA (dark green), also known as steroid receptor coactivator (SRC1), and/or CREB-binding protein (CBP) or the related protein p300 (dark pink), as well as the associated coactivator/HATs PCAF (dark blue) and PCIP (light pink). YY1 may also activate transcription through direct association with p300 [41], although it is not known whether HDAC and p300 associate with YY1 simultaneously.

Table 1

Summary of HDAC inhibitor effects on various genes and their relationship to transcriptional regulation by YY1.

Transcript (gene)	YY1 regulation		Deacetylase inhibitor response			Vitamin D response	
	Binding site	YY1 effect	Inhibitors tested	Response	Direct effect?	VDRE site	Response
<i>calbindin</i> *	Y**	↓	Butyrate	↑	N/A	Y	↑
<i>osteocalcin</i> †	Y	↓	N/A	↑††	N/A	Y	↑
<i>p21/waf1</i> *	Y**	↓	Butyrate, TPX	↑	Y	Y	↑
<i>cyclin D1</i> §	Y**	↑	Butyrate	↓	Y		
<i>c-fos</i> #	Y	↓	Butyrate, TSA	↑	Y		
<i>p-glycoprotein (MDR1)</i> ¶	Y**	↓	Butyrate	↑	N/A		
<i>metallothionein</i> *'	Y**	↓	Butyrate	↑	N/A	GRE	↑

The genes listed were generally selected because their mRNA levels are modulated by HDAC inhibitors in a direct fashion and they contain consensus YY1-binding sites in their promoters. The promoter of the gene listed either contains a confirmed or putative (**) YY1-binding site (Y indicates that the gene contains a site). Transcriptional repression is indicated by ↓ and activation is indicated by ↑ (††predicted, depending on whether or not the gene contains a confirmed YY1-binding site). A subset of genes also induced by vitamin D and defined VDRE (vitamin D response element) sites are noted. In the case of the *metallothionein* gene, a glucocorticoid response element (GRE), rather than a VDRE, overlaps the YY1 site [42]. Most genes listed are activated through the inhibition of HDAC, but *cyclin D1* is transcriptionally downregulated directly by HDAC inhibitors [43]. N/A, data not available; TSA, trichostatin A; TPX, trapoxin (see Figure 1). *See Figure 2b and [39], (Genbank number L11891). †For an analysis of the YY1/VDRE sites see [38]. ‡See Figure 2a,b and [27], (Genbank number U24170); p21 has been

shown to be directly induced by butyrate and is also trapoxin-inducible [20,34]. §A putative YY1 site in the *cyclin D1* promoter is located at -748/-742 (Genbank number L09054) [43]. #Nucleotides -63/-54 of the *c-fos* promoter have been shown to be the target of butyrate-mediated induction of *c-fos* transcription [44]. This same site has been shown to contain binding sites for YY1 and cyclic-AMP-response binding protein (CREB) [45]. CREB associates with CREB-binding protein (CBP) which is reported to be a histone acetyltransferase [15,16]. ¶A consensus YY1 site, corresponding to a previously mapped repressive element [46], is found -368/-360 upstream of the *MDR1* transcription start site (Genbank number X58723). Butyrate upregulates transcription of *MDR1* [47]. *Transcription of *metallothionein* is induced by butyrate [42]. A consensus YY1 site is found on the antisense strand from nucleotides -240/-230 (Genbank number D10551). This region overlaps a glucocorticoid response element (GRE; -245/-231) [48] providing the possibility for glucocorticoid receptor-mediated displacement of YY1.

these macromolecular complexes are recruited by nuclear receptors and by the Mad family of E-box DNA-binding transcription factors [6,15]. Interestingly, YY1 associates with the coactivator p300 [41], which has recently been shown to possess both an associated and an intrinsic HAT activity [15,16]. YY1 might therefore indirectly mediate both acetylation and deacetylation at certain promoters. It is unknown if YY1 associates with both p300 and HDAC simultaneously. Whether cells not expressing p21 will be affected by deacetylase inhibitors remains to be tested.

Conclusions and prospects

The potential anti-colon-cancer properties of fiber can be linked to a small-molecule signaling pathway involving butyrate, a by-product of fiber-metabolizing bacteria within the colon, and its cellular target HDAC. Butyrate inhibits the enzymatic activity of HDAC, resulting in changes in the transcription of specific genes, including the induction of the cyclin-dependent kinase inhibitor p21/Cip1/WAF1. Both inhibition of HDAC and expression of p21 result in cell-cycle arrest and differentiation, suggesting that induction of p21 is a pivotal event in HDAC inhibitor-mediated cell-cycle arrest. Experiments suggest that both targeted and non-targeted deacetylation of the p21 promoter by

HDAC may be important for the transcriptional repression of p21. We speculate that the DNA-binding transcription factor YY1 negatively regulates p21 in part by recruiting HDAC to a proposed YY1-binding site in the promoter of the p21 gene. Butyrate may therefore alleviate repression of p21 by inhibiting a YY1-targeted HDAC. The mechanism is similar to that used by steroid hormones in activating signaling pathways that lead to modified transcription of target genes. In both cases, a membrane-permeable small molecule binds to a protein involved in targeted gene regulation, thus altering the transcription of specific genes. The further study of the mechanism of HDAC inhibitor-mediated cell-cycle arrest could lead to the design of other potent small-molecule HDAC inhibitors with potential application in colon-cancer therapies.

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