

Cytolethal distending toxin: creating a gap in the cell cycle

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Cytolethal distending toxin (CDT) is a novel bacterial toxin that is produced by a variety of pathogenic bacteria. The mechanism of cytotoxicity of CDT is unique in that it enters into eukaryotic cells and breaks double-stranded DNA. This initiates the cell’s own DNA damage-response mechanisms, resulting in the arrest of the cell cycle at the G2/M boundary. Affected cells enlarge until they finally undergo programmed cell death. This review encapsulates recent work on CDT and focuses on the molecular mechanisms used by this toxin to block cell-cycle progression, the benefit to the bacterium of possession of this toxin and the clinical relevance of intoxication.

Introduction

Protein toxins are well-established vehicles of bacterial virulence and such toxins often have precise effects on normal host-cell function. Many act on membranes or interfere with intracellular signalling by interacting with specific signalling proteins, thus aiding in the process of infection. Cytolethal distending toxin (CDT) has a novel mechanism of action – interference with eukaryotic cell-cycle progression. The secret to this toxin’s action appears to be the nuclease activity of one of its subunits, which, by causing damage to chromosomal DNA, induces a block in the normal progression of cells through the complex signalling process known as the cell cycle.

CDT was first identified in culture supernatants from clinical isolates of Escherichia coli (Johnson & Lior, 1988a) and Campylobacter spp. (Johnson & Lior, 1988b), which were shown to induce cell distension and cytotoxicity in cultures of mammalian cells. Indeed, a wide range of cells have now been shown to be susceptible to CDT (Table 1). It is established that CDT is the product of a three-gene operon (cdtA, cdtB and cdtC) that produces proteins of 25–30 kDa (CdtA), 29–31 kDa (CdtB) and 20.7–21.2 kDa (CdtC). There is still some controversy over the exact number of CDT proteins needed to act on cells to produce intoxication (Shenker et al., 2000; Akifusa et al., 2001; Deng et al., 2001; Lewis et al., 2001; Saiki et al., 2001), which will be discussed later in the review.

Currently, it is established that CDT is produced by E. coli (Johnson & Lior, 1988a), Campylobacter spp. (Johnson & Lior, 1988b), Haemophilus ducreyi (Cope et al., 1997), Actinobacillus actinomycetemcomitans (Sugai et al., 1998) and Helicobacter hepaticus (Young et al., 2008). The recently sequenced genome of a Salmonella enterica serovar has revealed the presence of a cdtB gene in this organism, but no matching cdtA or cdtC genes (Parkhill et al., 2001), as the CdtB protein is transported into the mammalian cell by the internalization of the bacterium (Haghjoo & Galán, 2004). Four different forms of CDT have been identified in E. coli. The first to be discovered (termed EC I CDT) (Scott & Kaper, 1994) was from a clinical isolate of E. coli, 6468/62 (O86: H34). There is a newly described type IV E. coli CDT variant, isolated from various E. coli isolates of intestinal and extra-intestinal origin (Tóth et al., 2003). From a partial sequence of the cdtB gene, EC IV appears to have greatest similarity to EC I. EC I and IV have lower sequence identity to two other forms of CDT that are found in E. coli strain 9142-88 (O128: H1), known as EC II (Pickett et al., 1994), and in E. coli strain S5 (O15: H21), known as EC III, which carry the three cdt genes on a large virulence plasmid (Pérès et al., 1997).

The CDT family

Unravelling the mysteries of CDT is complicated by the large number of organisms that possess this toxin and the variation in the amino acid sequences of the individual toxins. The variation in sequence identity ranges from 21-6 %, between E. coli (III) CdtA and Campylobacter jejuni CdtA, to 96-8 %, between the CdtB proteins of A. actinomycetemcomitans and Haemophilus ducreyi. Examination of the homology of the CdtB amino acid sequences has suggested that the CDTs can be divided into two main groups: one consisting of E. coli CDTs and the other containing all toxins made by other bacteria, with Salmonella typhi as a common predecessor to both groups. The evolutionary origins of CDT are not understood, but evidence indicates that the cdt operon is transferred on mobile elements. The strongest evidence for this proposition is the presence of E. coli type III CDT on a pVir plasmid (Pérès et al., 1997) and from studies demonstrating cdt-flanking regions with homology to bacterioph-
Table 1. Cell populations that have been tested with CDT from various bacteria

EC, Escherichia coli types I–IV; Sd, Shigella dysenteriae; Hd, Haemophilus ducreyi; Aa, Actinobacillus actinomycetemcomitans; Cj, Campylobacter jejuni; Hh, Helicobacter hepaticus.

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<td>Svensson et al. (2001)</td>
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<td>Sensitive to CDT activity</td>
<td>Shenker et al. (2001)</td>
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<td>Sirc (rabbit cornea fibroblast like cells)</td>
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<td>Vero (African green monkey kidney cells)</td>
<td>Sensitive to CDT activity</td>
<td>Johnson &amp; Lior (1988b)</td>
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*Primary cells.
age P2 and lambda in some strains of *E. coli* O157 : H− and O157 : H7 (Janka et al., 2003). The cdt operons in *A. actinomycetemcomitans* and *Haemophilus ducreyi* are flanked by regions with homology to genes associated with integration of virulence plasmids (Mayer et al., 1999) and transposable elements (*Haemophilus ducreyi* sequencing project). Indeed, in *A. actinomycetemcomitans* CDT, there are two open reading frames (ORFs) upstream of the *cdt* genes. The first, ORF1, has sequence similarity to an integration site operon. Deletion of ORF1 had no influence on has been shown that both ORFs are cotranscribed with the al.

The mechanism of action of CDT on cell-cycle kinetics

The eukaryotic cell cycle is a masterpiece of cell signalling, synthetic biochemistry and intracellular movement, which is only visible at mitosis when the replicated chromosomes are taken into daughter cells. After this comes a variable period, depending on the cell type and its environment, called G (for gap) 1. With the appropriate growth signals, cells then begin to produce the machinery for replicating their DNA and enter into the synthesis (S) phase, where the DNA is replicated and cells end up with 4n DNA content. Between the end of DNA synthesis and mitosis is a second gap (G₂) in which, it is now appreciated, cells check the fidelity of their DNA replication before allowing progression into mitosis and the production of daughter cells. It was only in the late 1990s that CDT was found to exert its effects on cells by blocking the cell cycle at the G₂/M boundary (Comayras et al., 1997; Péres et al., 1997; Whitehouse et al., 1998). The obvious next question was: how does CDT exert this cell-cycle block?

Many factors contribute to controlling the cell cycle (Fig. 1). Two principal groups of controlling proteins are the cyclins and the cyclin–dependent kinases (termed Cdc2), which form complexes that are controlled, in turn, by other cell-cycle proteins (Smits & Medema, 2001). Entry into mitosis is controlled by the cyclin B1–cdc2 complex. In turn, cdc2 needs to interact with a phosphatase, cdc25, which removes phosphate groups from threonine at position 14 and tyrosine at position 15 in the protein. In cells exposed to CDT, a reduction in active cdc2 is observed (Comayras et al., 1997). This is not due to a reduction in cdc25 (Shenker et al., 2001) and it was speculated that the reduction in active cdc2 was due to activation of the G₂ cell-cycle checkpoint cascade, which prevents cell replication and is often caused by DNA damage (Sert et al., 1999).

The clue to the mechanism of action of CDT was the finding that CdtB has homology to type I deoxyribonuclease and could degrade DNA (Lara-Tejero & Galán, 2000). Was DNA damage, leading to activation of a checkpoint cascade, the mechanism of action of CDT? Ataxia telangiectasia-mutated (ATM) kinase is a tumour-suppressor protein that acts at an early stage in response to chromosomal DNA damage by stopping cell-cycle progression via checkpoint kinases (Chks) and by interacting with other tumour suppressors, such as p53 and BRCA1 (breast cancer 1), as well as activating DNA-repair factors. Involvement of the ATM protein in the response to CDT was demonstrated when ATM-deficient lymphoblastoid cell lines showed a slower response to CDT (Cortes-Bratti et al., 2001). CDT has been used in a number of studies to examine its DNA-damaging effect on various ATM-dependent pathways, such as activation of actin stress-fibre formation, which is a cellular response to genotoxic stress, occurs after activation of the G₂ phase, which itself is also activated by ATM.

**Biological activity of the CDT proteins and their interactions**

Our understanding of the mechanism of action of the individual CDT proteins and their requirement to interact, if any, to form a functional toxin is still unclear in some systems. Most of the literature would conclude that all three CDT proteins are required for toxic activity. However, there

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**Fig. 1.** Diagram of key DNA checkpoint pathways associated with CDT action. The ataxia telangiectasia-mutated gene (ATM) encodes a protein kinase that acts as a tumour suppressor. Mutation of the ATM gene causes the disease ataxia telangiectasia, which involves an inherited predisposition to some cancers. ATM activation by DNA damage from the deoxyribonuclease activity of CdtB stimulates DNA repair and blocks progression through the cell cycle. ATM interacts with a broad network of proteins, including checkpoint factors (chk1), which inhibit the dephosphorylation of the cyclin B–cdc2 complex indirectly via cdc25, thereby preventing the cell’s entry into mitosis. DNA-repair factors (Rad50, NBS1 and Mre11) are activated via phosphorylation of the histone H2AX by ATM. This complex promotes DNA double-strand repair (Li et al., 2002; Hassane et al., 2003). Stress-fibre formation, which is a symptom of genotoxic stress, occurs after activation of the G₂ phase, which itself is also activated by ATM.
are a number of papers that claim that cell-cycle inhibition can be achieved by individual CDT proteins or by only two of these proteins working in combination. The most striking is the series of papers reporting that the *A. actinomyctecomitis* CdtB is a potent inhibitor of the proliferation of human circulating T cells (Shenker *et al.*, 1999, 2000, 2001). It is possible that human T lymphocytes have an enhanced sensitivity to CDT and respond to individual components, such as CdtB. Other workers have reported that CdtB plus CdtC retains some cell cycle-inhibitory activity (Akifusa *et al.*, 2001; Deng *et al.*, 2001). Another report suggests that CdtA and CdtB can produce cell-cycle inhibition (Saiki *et al.*, 2001).

With three proteins capable of forming an active toxin, what combinations of protein binding are required to generate an active toxin complex? By using various immunological methods of assessing protein interactions, it appears that CdtB associates with CdtA, but only binds weakly, if at all, with CdtC in *Haemophilus ducreyi* (Pérès *et al.*, 1997) and *C. jejuni* (Lara-Tejero & Galán, 2001). Interestingly, by using gel filtration to assess complex size, when CdtA, B and C were mixed together, the mass of the complex was approximately 80 kDa (Lara-Tejero & Galán, 2001). The composition of this complex has not been defined, but this work shows that a stable complex can be produced by these toxin proteins. Interaction of the other two Cdt proteins with CdtC results in a major shift in the isoelectric point of the latter protein — the exact reason for this remains undefined (Frisk *et al.*, 2001).

What do each of the CDT proteins bring to the party? *A. actinomyctecomitis* CdtA has a signal peptidase II recognition site that is normally found on lipoproteins, suggesting that this protein may be anchored to the bacterial outer membrane (Mayer *et al.*, 1999); this has been confirmed for the CdtA from *Haemophilus ducreyi* (Lewis *et al.*, 2001). CdtA labelled with either a fluorophore (Mao & DiRienzo, 2002) or with biotin (Lee *et al.*, 2003) has been found to localize to selected areas of the plasma membrane of mammalian cells. The binding domain in CdtA is not defined, but all CdtA homologues have a ricin B lectin domain that is found in many proteins (lectins) that recognize oligosaccharides (Hazes & Read, 1995; Hirabayashi *et al.*, 1998). Plasma-membrane oligosaccharides are therefore the likely ‘receptors’ for CdtA, although this has yet to be formally proven.

Thus, the current hypothesis to explain the action of CDT would be that CdtA forms a complex with CdtB (and CdtC) to allow cell binding and, presumably, cell entry. Once inside the target cell, CdtB, which has nuclease activity (Elwell & Dreyfus, 2000; Lara-Tejero & Galán, 2000), targets to the nucleus by using a unique nuclear localization signal domain in the N terminus of the protein (Nishikubo *et al.*, 2003).

This leaves CdtC as the least understood of the CDT trios of proteins. Like CdtA, CdtC shares no homology with other proteins. As stated previously, most studies suggest that CdtC is required for the formation of active toxin (Akifusa *et al.*, 2001; Deng *et al.*, 2001; Frisk *et al.*, 2001; Lara-Tejero & Galán, 2001; Lewis *et al.*, 2001; Saiki *et al.*, 2001; Mao & DiRienzo, 2002). There is evidence that the CdtC of *C. jejuni* binds to cell membranes (Lee *et al.*, 2003) and unpublished studies from the authors’ group have shown that *A. actinomyctecomitis* CdtC can bind to cell membranes and a complex between CdtC and CdtB can enter into HEp2 cells. Scrutiny of the amino acid sequence of one subgroup of CDT proteins, consisting of the *E. coli* CDTs, shows that the CdtC proteins have a ricin B lectin domain, indicating that these proteins may also bind to oligosaccharides. A recent study that supports the involvement of CdtC in host-cell binding showed that *Haemophilus ducreyi* CdtA and CdtC can form a complex that, when pre-incubated with host cells, confers a protective response against the full CDT complex. However, cytotoxicity is established when CdtB is added alone (Deng & Hansen, 2003). This protective effect was not seen with pre-incubation of the individual Cdt A and CdtC proteins, implying that host-cell binding involves both proteins. Presumably, this means that the incomplete CDT complex occupied the available receptors for CDT, preventing the CDT holotoxin from binding.

Homologous domains of the CdtA and CdtC proteins from different species have been proposed to be involved in toxin-subunit binding to form the holotoxin, and not in binding of the toxin to host cells. If this is the case, then the non-homologous regions in CdtA and CdtC may be involved in host-cell binding and it is therefore possible that they may not bind to the same host-cell receptor (Lee *et al.*, 2003). The recent publication of the three-dimensional crystallographic structure of *Haemophilus ducreyi* CdtC (Nešić *et al.*, 2004) confirmed that the tripartite CDT complex consists of a DNase (CdtB) bound to two lectin subunits (CdtA and CdtC). A deeply grooved, highly aromatic surface exists in the region of the CdtA and CdtC complex that is not involved

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**Fig. 2.** Diagram illustrating internalization of CDT. CDT attaches itself to the cell membrane via a receptor and enters the cell by receptor-mediated endocytosis. CDT is then transported by the endosome to the cytosol through the ER, but this is unconfirmed. Once in the cytosol, CDT enters the nucleus with its unique nuclear localization signal and the deoxyribonuclease activity of CdtB results in DNA damage and subsequent apoptosis.
with binding CdtB and is critical for CDT activity. It is thought that this ‘aromatic surface’ is responsible for cell-surface binding. The three-dimensional structure also revealed that the N terminus of CdtC created a steric block of the active site of CdtB, inhibiting CdtB DNase activity. This seems to be a self-regulatory mechanism for CDT, possibly to prevent indiscriminate DNase activity.

Internalization of CDT

It is well-established that a variety of toxins (e.g. Shiga and cholera toxins) enter into mammalian cells by utilizing the endosomal-uptake pathways used by cells to transport material from the outside of the cell into the Golgi apparatus and the endoplasmic reticulum (ER) (Falnes & Sandvig, 2000). It has been established, by using a variety of inhibitors, that Haemophilus ducreyi CDT enters into HEp2 cells via clathrin-coated pits – a molecular mechanism for selectively concentrating extracellular macromolecules on receptors and taking them into cells. The CDT translocates to the Golgi apparatus (Cortes-Bratti et al., 2000) and ultimately the CdtB will move to, and enter into, the nucleus.

Clinical significance of CDT

Many bacteria possess CDT, suggesting that this toxin must confer some evolutionary advantage to the organism. However, what that advantage is remains to be defined. In E. coli, the role that this toxin plays remains unclear. Studies of E. coli isolates taken from diarrhoeal stools have shown that only a low percentage (0.5–3.1%) of E. coli strains are positive for cdt genes, although the percentage is higher than that in non-diarrhoeic isolates (0.5–1%) (Albert et al., 1996; da Silva & da Silva Leite, 2002; Marques et al., 2003). These studies do not show a strong association of CDT with diarrhoeal disease. However, there are reasons for suspecting an underestimate in detection of cdt genes, as not all E. coli CDT variants [such as the recently described variant EC IV (Tóth et al., 2003)] would have been tested in these early studies. CDT-positive strains of E. coli have also been implicated in neonatal bacterial meningitis (Johnson et al., 2003) and urinary tract infections (Starcic et al., 2002; Tóth et al., 2003). These findings suggest that CDT-positive E. coli may act as opportunistic pathogens and that the advantage conferred by CDT is not directed to intestinal infection. Of course, it is possible that CDT does not act alone, but in combination with the range of other toxins produced by E. coli, such as enterotoxin (da Silva & da Silva Leite, 2002) or cytotoxic necrotizing factor type 2 (Pérès et al., 1997).

Direct experimental evidence for a role of CDT in diarrhoeal disease comes from experiments performed by using a cloned Shigella dysenteriae cdt operon in non-diarrhoeic, recombinant E. coli. This CDT-positive recombinant E. coli was able to cause diarrhoea when administered to suckling mice (Okuda et al., 1997). Inactivation of CDT in C. jejuni resulted in the recovery of fewer bacteria from the blood, spleen and liver tissues of adult severe combined immunodeficiency (SCID) mice, demonstrating the role of CDT in Campylobacter spp. invasiveness (Purdy et al., 2000). A recent study on C. jejuni CDT mutants showed that, although bacteria lacking CDT were able to colonize NF-κB-deficient mice, they did not cause gastroenteritis like wild-type C. jejuni (Fox et al., 2004). Similar studies with Helicobacter hepaticus CDT mutants have shown that CDT is not required for colonization of the mouse gut, but contributes to lesion formation (Young et al., 2004). Two CDT-producing bacteria (Haemophilus ducreyi and A. actinomycetemcomitans, both members of the family Pasteurellaceae) have been the centre of much attention in recent years. The former bacterium causes the sexually transmitted disease ‘chancroid’, which is common in sub-Saharan Africa. This disease is characterized by genital ulcers. Studies of Haemophilus ducreyi isogenic mutants defective in CDT activity have shown that there is no difference in virulence or formation of lesions compared with the wild-type parental strain in the human and rabbit models of chancroid (Stevens et al., 1999; Lewis et al., 2001; Young et al., 2001). Although CDT does not appear to be involved in the development of chancroid lesions, it may contribute to the persistence of lesions by delaying the healing response. CDT has been shown to inhibit endothelial-cell proliferation in tissue culture and in an in vitro model of angiogenesis (Swensson et al., 2002). A. actinomycetemcomitans is an oral pathogen that is associated with localized aggressive periodontitis and implicated in non-oral infections, such as endocarditis, meningitis, osteomyelitis and glomerulonephritis (Collazos et al., 1999; Slots & Ting, 1999; Henderson et al., 2003). This organism is unique amongst the currently identified periodontopathogenic bacteria in producing CDT (Yamano et al., 2003), but the role of CDT in periodontal disease has not been established. There is a low occurrence of A. actinomycetemcomitans strains possessing the cdt genes in patients with periodontitis, but there is a strong association between possession of the cdt genes and the aggressive form of this disease (Tan et al., 2002). Two studies have found that a high percentage of A. actinomycetemcomitans strains taken from culture collections and clinical isolates possess CDT. Both studies reported variability in the toxin titre amongst strains (Ahmed et al., 2001; Fabris et al., 2002). The potential roles of the A. actinomycetemcomitans CDT are not clear. It may be acting directly on the gingival epithelium to breach this barrier and/or on underlying immune cells. The possibility that it may synergize with the other major toxin, the leukotoxin (Lally et al., 1989), of this bacterium needs to be investigated.

Effects of CDT on the immune system

Cell cycle-blocking toxins can only have effects on dividing cell populations and the more rapidly dividing the cell population, the more significant the action of the toxin will be. Acquired immunity is absolutely dependent on the rapid proliferation of antigen-specific T and B cells following exposure to antigens (such as a bacterium), to provide
sufficient effector cells to kill the invading pathogen and neutralize its toxins. Is there any evidence that CDT is immune-suppressive?

Experiments using the Jurkat T-lymphoblastic leukaemia cell line were the first to show that *Haemophilus ducreyi* CDT influences the acquired immune response by inhibiting proliferation of T cells (Gelfanova et al., 1999). This was confirmed by Svensson et al. (2001), who demonstrated that purified mitogen-induced proliferation of IgM- and IgG-antibody-producing B cells was also affected by CDT.

However, CDT’s effects on the immune system do appear to be limited, as each Cdt component (CdtA, CdtB and CdtC) and the holotoxin itself can elicit an antibody response in immunized rabbits (Wising et al., 2002). Therefore, CDT may not be able to suppress antibody production completely, but may only dampen it. In addition, the innate immune response is not affected by *Haemophilus ducreyi* CDT, as monocytes and polymorphonuclear leukocytes are not sensitive to CDT (Svensson et al., 2001).

As has been described, there is some controversy over the requirement for the participation of all three CDT proteins in cell intoxication. Shenker and co-workers (Shenker et al., 1999, 2000, 2001) have reported that the CdtB protein of *A. actinomycetemcomitans* is sufficient to induce G2/M arrest and subsequent apoptosis of activated CD4+ and CD8+ T cells. However, CdtB alone, when tested on other cell types, is not sufficient to cause cell-cycle arrest (Akifusa et al., 2001; Deng et al., 2001; Lewis et al., 2001; Saiki et al., 2001) unless administered to the cell by microinjection (Lara-Tejero & Galán, 2000) or by using a lipid-based protein-delivery agent (Mao & DiRienzo, 2002). Therefore, CdtB must be able to enter into T lymphocytes by a novel uptake pathway.

The ability of CDT to elicit a cytokine response has also been investigated. Cytokines are important mediators in the immune response and are usually responsible for many of the clinical symptoms of disease. *C. jejuni* is known to elicit the secretion of the neutrophil chemokine interleukin 8 (IL8), which in itself is responsible for many of the clinical symptoms of *Campylobacter* enterocolitis, and it has been found that such IL8 release is dependent on CDT (Hickey et al., 2000). The cytokine response to *A. actinomycetemcomitans* CDT was investigated by using peripheral blood mononuclear cells and each component of the *A. actinomycetemcomitans* CDT was able to induce secretion of IL1β, IL6 and IL8, but to different extents and with evidence of synergism between the individual CDT proteins, particularly in the case of gamma interferon synthesis (Akifusa et al., 2001). However, one study has shown that *Haemophilus ducreyi* CDT is able to inhibit the cytokine response to some extent, by preventing ‘antigen presenting cell’ proliferation (Xu et al., 2004).

**Summary**

CDT is a toxin with a novel mode of action on the eukaryotic cell cycle. The potential actions of this toxin are: (i) inhibition of epithelial-cell proliferation and apoptosis, enabling the invasion of bacteria; (ii) inhibition of proliferation of cycling immune cells, resulting in local immunosuppression; and (iii) inhibition of the fibrotic response, which can wall off invading bacteria. Considering the clinical evidence of CDT’s involvement in pathogenesis, it appears that, although CDT’s ultimate function is to inhibit cell division, this ability may confer different virulence characteristics to different species of bacteria. For example, CDT aids the invasiveness of *Campylobacter* spp. into host tissues, whereas *Haemophilus ducreyi* CDT appears to delay healing by affecting the immune response and tissue regeneration. Whether this applies to the other CDT-possessing member of the family *Pasteurellaceae* (*A. actinomycetemcomitans*) is yet to be determined. The benefit of CDT to *E. coli* is also unclear; considering that many of the strains harbouring CDT are known to cause diarrhoea, it would be plausible that *E. coli* CDT-producing strains benefit by aiding invasiveness of the bacterium into tissues, as it does for the other diarrhoeic CDT-harbouring bacterium, *C. jejuni*. However, CDT-positive *E. coli* has been implicated not just in diarrhoeic disease, but also in urinary tract infections and bacterial meningitis. CDT seems be a versatile bacterial toxin that is utilized by a variety of pathogenic bacteria.

Whilst this review has highlighted the negative side of CDT, its activity may have some positive benefits. Consider that the therapeutic control of the cell cycle is a key goal in cancer chemotherapy. Further, consider that there is emerging evidence linking bacteria with cancer induction (Lax & Thomas, 2002). Is it too far-fetched to think of bacteria having pro- or anticancer actions and could CDT have some therapeutic use in cancer treatment?

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