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NEW DRUGS

DT-diaphorase: a target for new anticancer drugs

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Summary DT-diaphorase (DTD) is an obligate two-electron reductase which bioactivates chemotherapeutic quinones. DTD levels are elevated in a number of tumour types, including non-small cell lung carcinoma, colorectal carcinoma, liver cancers and breast carcinomas, when compared to the surrounding normal tissue. The differential in DTD between tumour and normal tissue should allow targeted activation of chemotherapeutic quinones in the tumour whilst minimising normal tissue toxicity.

The prototypical bioreductive drug is Mitomycin C (MMC) which is widely used in clinical practice. However, MMC is actually a relatively poor substrate for DTD and its metabolism is pH-dependent. Other bioreductive drugs have failed because of poor solubility and inability to surpass other agents in use. RH1, a novel diaziridinylbenzoquinone, is a more efficient substrate for DTD. It has been demonstrated to have anti-tumour effects both in vitro and in vivo and demonstrates a relationship between DTD expression levels and drug response. RH1 has recently entered a phase I clinical trial in solid tumours under the auspices of Cancer Research UK.

Recent work has demonstrated that DTD is present in the nucleus and is associated with both p53 and the heat shock protein, HSP-70. Furthermore, DTD is inducible by several non-toxic compounds and therefore much interest has focussed on increasing the differential in DTD levels between tumour and normal tissues.

Introduction

The enzyme DTD was first described in 1958 by Ernster and Navazio.[1] DTD was isolated from soluble rat liver homogenates and was shown to catalyse the oxidation of the co-factors nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) equally well. Subsequently the structure, mechanism of action and interactions of DTD have been investigated. During this time DTD has been referred to by a variety of names including NAD(P)H:quinone oxidoreductase (NQO) (EC 1.6.99.2), vitamin K reductase, phylloquinone reductase, menadione reductase, azo dye reductase, X-ray inducible transcript 3 (Xip3) and nicotinamide menadione oxidoreductase but, in keeping with current literature, the term "DT-Diaphorase" will be used throughout this review.

DTD bioactivates chemotherapeutic quinones, which are referred to as bioreductive agents. There

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is often significant over-expression of DTD in neoplastic tissue compared to the surrounding normal tissues. As a result of this, DTD has attracted considerable attention as a potential candidate for targeted anticancer therapy.

Many comprehensive reviews have already been published about DTD and bioreductive drugs.[2–7] This review seeks to discuss the general background and include more recent developments.

Structure, distribution and mechanism of action

DTD is a flavoprotein which exists as a dimer with each unit having a molecular weight of 32,000 kD and being associated with a flavin adenine dinucleotide (FAD) group, noncovalently attached to the protein. DTD is predominantly cytosolic with over 90% being found in the cytoplasm.[8] However, earlier studies showed DTD in the endoplasmic reticulum,[9] mitochondria[10] and Golgi body[11] and recent work has shown that DTD is present in the nucleus.[12]

DTD is an obligatory two electron reductase enzyme. This is in contrast to the one electron reductase enzymes such as NADH:cytochrome *b* reductase, NADPH:cytochrome P450 reductase and xanthine oxidase. DTD functions via a "ping-pong" kinetic mechanism (cofactor binds on and then off followed by substrate on and then off) utilising the co-factors NADH and NADPH equally well as the electron donor.[13]

DTD has multiple cellular roles, many of which are poorly understood. In this review, we are most interested in the reduction of benzoquinones to hydroquinones, especially cytotoxic quinones, but it is also involved in reduction of dietary and environmental quinones. DTD also acts as a Phase II detoxification enzyme with the detoxifying step bypassing the formation of free radicals and so protecting tissues against mutagens, carcinogens and cytotoxics. DTD can recycle the membrane antioxidants ubiquinone and vitamin E.[14,15] DTD is induced in a "stress response", for example in hypoxic conditions, along with other enzymes, including the glutathione S-transferases which conjugate hydrophobic electrophiles and reactive oxygen species, UDP-transferases which catalyse the conjugation and thus excretion of xenobiotics, epoxide hydrolase which inactivates epoxides and γ-glutamylcysteine synthetase which regulates glutathione metabolism.[16]

DTD is widely distributed in animals, plants and bacteria. In humans, DTD is distributed throughout the body with highest levels in epithelial and endothelial cells, especially of the kidney and gastrointestinal tract.[17,18] The amino acid sequence has been determined for rat, mouse and human DTD.[19] The amino acid sequence of mouse DTD is more homologous to the rat enzyme than the human enzyme. However, rat DTD has faster reductive activation than mouse DTD due to a base difference at residue 104 which is critical for catalytic activity.[19] In addition, DTD has high levels of activity in extrahepatic tissues in humans but is most abundant in the liver of other mammals such as rat, monkey and dog. Mouse has less DTD in the liver than other animals and this, in combination with the similar reductive rate to humans, means that mouse models may be more comparable to humans than those of rats or other species.

Gene

DTD activity is encoded by four genetic loci known as NQO1, NQO2, NQO3 and NQO4.[20] The majority of DTD is coded for by the NQO1 gene but as yet the roles of the other three are poorly understood. NQO1 is a single copy gene located on chromosome 16q22.1 and encodes for a protein of 274 residues.[21] NQO1 consists of 6 exons and 5 introns and spans a 20 kilobase region. Exon 1 codes for the 5′UT region whilst exons 2—6 code for the remainder of the gene and the 3′UT region. The sixth exon has four potential polyadenylation signal sequences, three of which are used. A single copy of human Alu repetitive sequence is sited between the second and third polyadenylation sites. Jaiswal et al.[22] have sequenced the region upstream of the protein coding sequence for DTD. They have identified several *cis*-activating transcriptional activators, including an antioxidant response element (ARE), within which a perfect AP1 site resides along with a xenobiotic response element (XRE), an AP2 element, a CAT box, a TATA box and a NF-κB binding site.[22–24] Jun, fos and other novel proteins from the nucleus, including oestrogen receptors,[25] bind to the ARE and mediate signal transduction. The molecular biology of DTD expression has been extensively reviewed by Jaiswal.[16]

A second gene product, NQO2 (NRH:quinone oxidoreductase) has been described. The role of NQO2 in the detoxification of quinones is unknown. The NQO2 gene is located on chromosome 6p25 and the protein product has 231 residues (43 amino acids shorter than NQO1 at the carboxyl terminal end). The initiation codon of the NQO2 cDNA aligns perfectly with the initiation codons of human and rat NQO1 cDNAs. NQO1 and NQO2 are homologous

for 54% of cDNA and 49% of protein. NQO2 is expressed in fewer tissues (including heart, lung, brain and skeletal muscle) than NQO1. NQO2 and NQO1 differ in their cofactor requirement with NQO2 using dihydronicotinamide riboside (NRH) rather than NADH or NADPH as the electron donor. NQO2 is resistant to typical inhibitors of DTD such as dicumarol. Readers are referred to the reviews by Jaiswal, Chen and Long for more detailed discussion of NQO2.[26–28]

Polymorphism

Traver et al.[29] have demonstrated a single point nucleotide polymorphism (SNP), known as NQO1*2, in the NQO1 sequence of the BE colorectal cell line, which has no detectable DTD activity. A homologous base substitution (cytosine to thymidine) at position 609 on the cDNA sequence results in a proline to serine substitution in the protein. The inactive DTD expressed is much less stable than wild-type DTD. Wild-type DTD persists in cells for over 18 h whilst the mutant protein is broken down via the ubiquitin proteasomal pathway (UPP) in just 1.2 h.[30] This SNP appears to cause a change in the enzyme conformation so that there is a decrease in the FAD binding affinity and thus a loss of enzyme activity.[31] There is ethnic variation in the occurrence of this SNP with 4.4% of the Caucasian population and 20.3% of Asians having the homologous genotype.[32] Heterozygotes for this SNP have intermediate activity compared with people with the wild type.[33] Multiple further aberrations of DTD have been reported but the physiological significance of these have not yet been determined. These include the 465C>T variant (NQO1*3) which is associated with increased alternative splicing and decreased expression of DTD.[34,35]

Localisation

Winski et al.[12] recently confirmed the presence of DTD in the nucleus. This is of interest when we consider the bioreduction of cytotoxic agents as it would allow the drug to be activated in the nucleus itself. The process of nuclear localisation can occur by either passive diffusion through nuclear pores, active transport by nuclear localisation signals or binding to chaperone proteins. The DTD molecule is too large for passive diffusion, its diameter is 60—70 nm and would need to be less than 10 nm.[36] The classic nuclear localisation signal required for active transport is not present in the DTD molecule. Therefore DTD is thought to bind to molecular chaperone proteins, such as HSP-70. HSPs are involved in protein synthesis and folding, vesicular trafficking, and antigen processing and presentation. HSP-70 binds to hydrophobic regions of unfolded proteins which go through ATP-dependent binding cycles and the nascent protein is manipulated into an active conformation. Using co-immunoprecipitation, it has been shown that HSP-70 associates with wild-type DTD, and thus stabilises it, but not with the mutant DTD.[37] Anwar et al.[37] have generated a plasmid containing a DTD coding region with a mutated HSP-70 binding site. This plasmid did not associate with HSP-70, was catalytically inactive and was degraded by the UPP. DTD itself has been implicated in the stabilisation of the tumour suppressor gene, p53, which is responsible for regulating cell cycle G1 arrest and for activating apoptosis. Wild-type DTD does interact with p53 but the cellular role of this interaction is not yet known. DTD-mediated reduction of quinones induces p53[38] and this reduction, and subsequent p53 induction, can be suppressed using the specific DTD inhibitor, dicumarol.[39,40] Comparison of inhibitors of DTD and inhibitors of another HSP, HSP-90 indicates that DTD and HSP-90 act through different mechanisms to induce p53 degradation and thus suppress apoptosis.[40]

DTD and cancer

DTD is over-expressed in many cancerous tissues compared to normal tissue, especially non-small cell lung carcinoma (NSCLC) (Table 1).[18,41–53] Since DTD acts to detoxify and protect the cell from toxins and mutagens, it may be that upregulation of DTD is part of the early carcinogenic process. This may involve epigenetic factors such as hypomethylation of the NQO1 gene,[54] elevation of the jun and fos proto-oncogenes,[55] altered promoter function, altered repressor function or post-transcriptional modifications.[5]

All four histological sub-types of NSCLC exhibit an increase in enzyme expression and activity, especially tumours that are well-differentiated, stage I and II and epidermoid.[53] Small cell lung carcinomas (SCLC) do not have raised DTD activity.[50] However, when SCLC cell lines are transfected with the v-Ha-ras proto-oncogene, thus causing a transition to the NSCLC phenotype through an increase in AP1 activity,[56] DTD activity is increased.[50] Some workers have reported that DTD activity is highest in the lung tumours of those who have never smoked or who have stopped smoking. This trend is not replicated for colon tumours.[18] Conversely, those lung cancer patients

Table 1 Ratio of DTD activity in tumour tissue compared to normal tissue

Cells	Ratio	References
Rat leydig cell tumours	2.5—7.7	41
Rat hepatomas	2—2.4	42
Hyperplastic rat liver nodules	6.0	43
Mouse liver tumours	1.5—2.2	44
Rat brain tumours	10.8	45
Rat ascites hepatoma	4.7	46
Human colon carcinoma primary	2.5—3.9	18,47,52
Human colon carcinoma metastases	4.7	52
Human breast carcinoma	NS—9.5	18,48,53
Human NSCLC	8.2—19.2	18,50,52,53
Human SCLC	0.25	50
Human liver carcinoma	3.8—50	18,49
Human stomach carcinoma	0.39	18
Human kidney carcinoma	0.1—0.3	18,51
Human head/neck (squamous)	1.07	52

NS: not significant.

who are alcohol users have significantly higher DTD activity in their tumours than those who did not; again this trend has not been replicated for those with colon tumours.[18] These differences in the lung tissue of smokers were not observed in the work of Marin et al.[53]

Other tumours which over-express DTD include colorectal, liver and breast carcinomas but increased expression is not seen with all tumour types. For example, stomach and kidney tumours have lower DTD activity than the surrounding normal tissue, and head and neck tumours have the same DTD activity as the surrounding normal tissue.[51,52] Some tissue types have high baseline activity levels, such as stomach and kidney, and it may not be possible to increase the DTD activity further. There may also be marked variation of DTD activity within a specific tumour type[50] and possibly within an individual tumour. Advanced metastatic tissue has been found to have greater expression and activity of DTD than tumours without metastases and poorly differentiated tumours have higher activity than well-differentiated ones.[41,57] However, others have found lower absolute DTD activity in lymph node and liver metastases than in the primary tumour.[52] High expression and activity of DTD are typically found in "normal" hepatic tissue surrounding hepatic tumours.[46,49] It should be noted that DTD protein levels do not necessarily correlate with DTD activity.

DTD may play a role in cancer prevention so, conversely, it would be expected that individuals with the NQO1*2 SNP, and thus no active DTD, would have a higher susceptibility to developing cancer. Several studies have shown a correlation between NQO1 polymorphism and cancer incidence. Kidney tumour, lung cancer, colorectal carcinoma and oesophageal and gastric cancer patients all have a higher percentage of the NQO1 polymorphism than the normal population.[58–61] This correlation has not been shown in other studies with kidney, lung and colorectal cancer patients[62–65] or in patients with adult glioma,[66] Hodgkins Disease,[67] non-Hodgkins lymphoma[67] or prostate cancer.[68] Lack of DTD is associated with an increased risk of developing adult leukaemia[69] and paediatric leukaemia.[70,71] In cancer patients, the NQO1 polymorphism may be associated with an increased risk of chemotherapy-related myeloid leukaemia, including those leukaemias associated with abnormalities in chromosomes 5 and 7[72] but others disagree.[73] In addition, individuals with absent DTD activity do have increased susceptibility to bone marrow suppression after environmental exposure to benzene and benzene-like compounds.[74] In NQO1 knock-out mice, no detectable change in phenotype is observed and they appear indistinguishable from wild-type mice, however they are more susceptible to quinone toxicity[75] and have increased sensitivity to the development of skin and visceral tumours.[16]

Role in anticancer therapy

Paradoxically, the detoxifying enzyme DTD can activate certain xenobiotics, such as MMC, orthonaphthoquinones and aziridinylbenzoquinones.[76–78] This is of interest since drugs that are effectively bioactivated by DTD may allow tumour cytotoxicity without corresponding high levels of toxicity to normal tissues (see review by Workman,[55]). Reduction of quinones by DTD may lead to either production of a reactive alkylating species by rearrangement, or hydroquinones may auto-oxidise leading to the production of reactive oxygen species and toxicity. The reductive cycle can continue along both pathways unless the system becomes anaerobic at which time oxygen radical production decreases and the semiquinone can accumulate.[2] A correlation exists between the cytotoxicity of these bioreductive compounds, DTD activity, an increase in the pro-apoptotic BAD protein and the induction of apoptosis.[79] Although, bioreductive quinones are more active in tumour cells that over-express DTD, there is still some effect on cells without DTD, presumably due to single electron enzymatic reduction.[80]

Mitomycin C

Mitomycin C (6-Amino-8-{([aminocarbonyl]oxy) methyl}-1,1a,2,8,8a,8b-hexahydro-8-methoxy-5-methyl[1aR-(1aα,8β,8aα,8bα)]azirino(2′,3′:3,4)-pyrrolo-(1,2-α)indole-4,7-dione) is a naturally occurring anti-tumour quinone derived from *Streptomyces caespitosus*, a strain of actinomyces. It has been used as a cytotoxic since the 1960s and has activity against lung, stomach, head and neck, prostate, breast and bladder tumours. The dose-limiting toxicity of MMC is myelosuppression. MMC is the prototypical bioreductive agent as it is inactive until reduced by one-electron reductases or DTD.[81] Reduction allows DNA cross-links and strand breaks to form, thus inhibiting DNA replication. MMC sensitivity has been shown to be associated with high DTD levels by some workers but others have demonstrated discrepancies between in vitro and in vivo cytotoxicity.[29,50,78,82–87] Relating MMC cytotoxicity to DTD levels is complicated by the fact that MMC is a relatively poor substrate for DTD, is also bioactivated by one-electron reductases, activation is pH-dependent and may be influenced by hypoxia.[87,88] It has become evident that superior substrates for DTD exist and there has been much effort to produce novel agents that will target DTD more effectively.

E09

The indoloquinone E09 (3-hydroxy-5-aziridinyl-1-methyl-2(1H-indole-4,7-dione)-prop-beta-en-alpha-ol) is a synthetic analogue of MMC. EO9 is a moderately improved substrate for DTD when compared to MMC as it can redox cycle more effectively and is not pH-dependent. A strong association has been described between elevated DTD activity and EO9 sensitivity.[81,83, 89] EO9 entered clinical trials on the basis of its distinct anti-tumour profile and lack of myelosuppression. However, EO9 failed to produce any clinical responses and this has been attributed to a combination of rapid plasma clearance and dose-limiting kidney toxicity.[90]

CB1954

CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) is a nitrophenylaziridine which is cytotoxic to rat carcinoma cells which over-express DTD.[91] However, CB1594 is bioactivated much more slowly by human DTD than by rat DTD which has profound implications on its clinical utilisation.[92] Human tumour cells require the NQO2 gene, which is normally latent, to be activated by the synthetic co-factor NRH before CB1954 can be used in a cytotoxic manner.[93]

AZQ

AZQ (2,5-bis(carboethoxyamino)-3,6-diaziridinyl-1,4-benzoquinone) is an aziridinylbenzoquinone with good solubility. AZQ does not readily alkylate DNA at physiologically relevant pH but has a relatively high reduction potential and is easily reduced by both one electron reductases and DTD to form DNA alkylating species.[94] AZQ activation is inhibited by the DTD inhibitor, dicumarol.[94] A correlation is seen between DTD levels and cytotoxicity with AZQ.[95] AZQ underwent clinical trials, activity was demonstrated against progressive gliomas[96] and refractory lymphomas[97] but it was not able to surpass agents already in use.

BZQ

BZQ (2,5-bis(2-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone) is a powerful cross-linking aziridinylbenzoquinone which does not easily undergo reduction by either one or two electron reductases. BZQ is much less stable than AZQ and can readily alkylate in the absence of reduction[94] and its cytotoxicity is unaffected by the addition of dicumarol.[80]

MeDZQ

MeDZQ (2,5-diaziridinyl-3,6-dimethyl-1,4-benzoquinone) is the lead compound in the aziridinylbenzoquinone series. MeDZQ is an excellent substrate for DTD[95,98] and, after reduction by DTD, forms cross-links at GNC sequences.[99] There is a strong correlation between DTD levels in NSCLC cell lines and the cytotoxicity of MeDZQ.[87] However, the development of MeDZQ was limited by its poor solubility.

RH1

The novel diaziridinylbenzoquinone RH1 (2,5-diaziridinyl-3-hydoxymethyl-6-methyl-1,4-benzoquinone) (Fig. 1) may have advantages over existing bioreductive agents as it has good solubility, is an excellent substrate for DTD and antitumour effect has been demonstrated both in vitro and in vivo. RH1 is more soluble in water

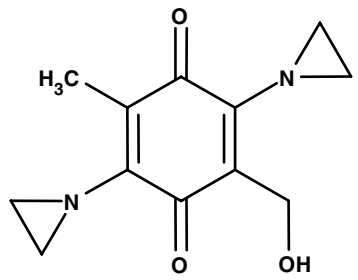

Figure 1 RH1 molecule, with two aziridine rings and hydroxyl group.

than MeDZQ due to its hydroxyl moiety. RH1 is rapidly taken up by cells and undergoes aziridine ring opening, which is enhanced by the presence of DTD, and causes formation of DNA adducts, particularly at GCC sequences.[100] The products of reduction by RH1 are more stable, less likely to be involved in redox cycling and less likely to be influenced by hypoxia than those of MMC.[101] RH1 is cleared ten-fold slower from the plasma than EO9.[102] In keeping with its mechanism of action RH1 has been shown to be significantly more active in cells that over-express DTD, naturally or through transfection, than in cell types with low DTD.[102,103] RH1 is an excellent substrate for DTD and is 50% more active than MeDZQ and 225 times better than MMC in cell lines.[103] RH1 has undergone in vivo efficacy testing by the Developmental Therapeutics Program of the National Cancer Institute and has demonstrated significant activity in both NSCLC and ovarian xenograft models.[104] Based upon the entire toxicology data in mice, rats and dogs, a starting dose level of 40 $\mu g/m^2$ daily for five days was suggested for human trials based upon this being 1/10th of the maximum tolerated dose in the most sensitive species, the dog. The mouse may be a better model as mouse and human liver have low levels as DTD whereas rat, monkey and dog have substantial amounts. Toxicity seen has included myelosuppression and local injection site inflammation. The lower renal metabolic rate seen with RH1, compared with EO9, suggests that RH1 may cause less renal toxicity.[102] RH1 formulated in 20% hydroxypropyl-β-cyclodextrin is associated with a reduction in injection site toxicity compared to its formulation in water without a detrimental effect on stability and this has been used as the drug delivery vehicle in subsequent studies.[104] RH1, diluted in cyclodextrin and given once a day for five days, has entered a phase I dose escalation study in human patients with solid tumours, under the auspices of Cancer Research UK.

Manipulation of DTD

Much is known about the inhibition of DTD by dicumarol. Of more interest are the selective induction of DTD in tumour tissue to increase tumour cell kill whilst minimising normal tissue toxicity and potential mechanisms of resistance to RH1 and whether they can be overcome.

Inhibition

DTD is very sensitive to the inhibitory effects of the anti-coagulants, dicumarol and warfarin.[105] Dicumarol binds with the FAD group and competitively inhibits DTD with respect to the nicotinamide coenzymes.[106] DTD is also competitively inhibited by Cibacron blue, chrysin, 7,8-dihydroxyflavone and phenidone. Recently, a potent inhibitor of DTD, ES936 (5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione) has been described.[107]

Induction

DTD expression in tumour cell lines can be induced by a variety of structurally different agents, including the thiol, 1,2-dithiol-3-thione (D3T); oltipraz (5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione), a synthetic D3T analogue that was used to treat schistosomiasis; extracts of cruciferous vegetables (brocolli and green onions) such as fisetin and quercetin, two flavenols; dietary antioxidants, such as 2(3)-tert-butyl-4-hydroxyanisole (BHA); benzenes; azo dyes; aspirin-like drugs (ALD); dioxin; diphenols, anti-oestrogens, glucocorticoids and isothiocyanates[108–126] (Table 2). DTD is also induced by hypoxia,[127] light[128] and the addition of some chemotherapeutic agents, including MMC and doxorubicin.[125,129] There are several comprehensive reviews on induction including that by Begleiter et al.[130]

Induction of DTD is mediated via an ARE, which is essential for the co-ordinated induction and expression of DTD. The AP1 site in the ARE is sensitive to induction by a number of agents and hypoxia, however AP1 binding proteins may not be necessary in all instances for ARE activation. Over-expression of c-fos or FRA1 represses binding to the ARE. Disruption of c-fos can increase DTD activity suggesting that c-fos is negatively regulating DTD.[3,16] It has been hypothesised[16] that Nrf2 is bound to Keap1, a repressor protein, and is retained in the cytoplasm. Dissociation of the Nrf2-keap1 dimer allows free Nrf2 to enter the nucleus where it heterodimerises with c-jun and binds to ARE. In-

terestingly Kepa et al.[131] have reported that in NSCLC, FRA1 and FRA2 along with jun-b bind to the AP1 site thus enabling induction of DTD. Conversely in SCLC, FRA1/FRA2 and jun-b appear to be absent which could explain why SCLC does not over-express DTD whilst NSCLC does. There is also evidence of involvement in induction by the NF-κB response element in the 5′ flanking region of the DTD gene.[24] Others have shown that the extracellular signal regulated protein kinase (ERK) pathway negatively regulates ARE induction.[132] It is felt that unknown cytosolic factors are involved in the

Table 2 Levels of DTD induction (induced value/baseline value)

Inducer	Target	Ratio	Reference
D3T	Murine lymphoma	22	108
	Murine bone marrow	2—3	109
	Human leukaemia	2-10	110—112
	Human head & neck cancer	1.3	111,112
	Human lung cancer	1.1—8.9	111,112
	Human colon cancer	NS—2.5	111,112
	Human breast cancer	NS—9.9	111,112
	Human ovarian cancer	1.5—2.7	111
	Human prostate cancer	NS—2.6	111,112
	Human skin cancer	1.2—1.4	111
	Human liver cancer	NS	111,112
	Human stomach cancer	1.7—3.3	111
	Human bone marrow	10.5	111
	Human kidney	3.5	111
	Human lung	2.4	111
D3T/MMC	Human NSCLC	2.5	113
	Human breast cancer	1.8—3.6	113
	Human colon cancer	2.3	113
	Human melanoma	2.3	113
	Human gastric cancer	1.9	113
	Human marrow	6.7	113
Oltipraz	Murine cell lines	1.5—3	114
	Rat liver post-aflatoxin	2.2—7.1	115
	Human colorectal cancer	2.0	116
Fisetin	Murine hepatoma	2.6	121
Quercetin	Human breast cancer	2.0	123
Retinoic acid	Human breast cancer	2.2	113
DMM	Human breast cancer	4.4	113
DMM/MMC	Human breast cancer	2.9	113
DMF	Human breast cancer	4.6	113
	Human lymphocytes	6.5	118
PG	Human breast cancer	2.9	113
PG/MMC	Human breast cancer	1.8	113
Sulforaphone	Murine hepatoma	6.0	117
	Murine liver and gut	1.5—3.1	119,124
	Human hepatoma	5.9	117
	Human breast cancer	0.4—9.4	113,117
	Human prostate cancer	1.8	117
	Human cervical cancer	3.9	117
	Human colon cancer	10.3	117
BHA	Murine liver	10.0	17
	Murine kidney	4.2	17
	Murine lung	3.6	17
	Murine small intestine	2.7—6.2	17,124
tBHQ	Murine hepatoma	5.3	117
	Human hepatoma	6.4	117
	Human breast cancer	0.6—10.0	117
	Human prostate cancer	3.0	117
	Human cervical cancer	4.3	117
	Human colon cancer	10.8	117

Table 2 (*continued*)

Inducer	Target	Ratio	Reference
β-naphthoflavone	Murine hepatoma	8.5	117
	Human hepatoma	6.3	117
	Human breast cancer	0.5—12.5	117
	Human prostate cancer	1.4	117
	Human cervical cancer	4.0	117
	Human colon cancer	9.5	117
	Human lymphocytes	1.5	118
Methyl vinyl sulfone	Human lymphocytes	3.9	118
Aspirin	Human breast cancer	1.5	113
Ibuprofen	Human breast cancer	NS	113
ALD	Human T lymphocytes	2.0—4.0	120
Anti-oestrogens	Murine hepatoma	1—2.9	122
Doxorubicin	Murine mammary	1.4	125
	Murine bone marrow	NS	125

ALD: aspirin like drugs, BHA: 2(3)-tert-butyl-4-hydroxyanisole, DMF: dimethyl fumarate, DMM: dimethyl maleate, MMC: mitomycin C, NS: not significant, PG: propyl gallate, tBHQ: tert-butylhydroquinone.

stimulation of Nrf and Jun binding to the ARE and that these may be kinases, phosphatases or redox proteins.[16]

Most work has been undertaken on D3T which is an effective monofunctional inducer (it elevates phase II enzymes selectively), both in vitro and in vivo.[115] Oltipraz is an analogue of D3T that has been shown to act as a chemoprotective agent in vivo and is now being assessed in clinical trials.[133] In this instance, we are interested in oltipraz as an inducing agent in established tumours. Dose—response curves of D3T and oltipraz have indicated that D3T was 15-fold more potent as an enzyme inducer than oltipraz,[134] and indeed D3T is superior to all the other analogues against which it has been compared. However, in practice, there are differences between the effects of inducers on murine and human cell lines[111] and oltipraz may have less side-effects in patients than D3T although this remains to be tested.

The differential between normal and neoplastic tissue is maintained, or even accentuated, when DTD is induced by D3T. In murine lymphoma cells, D3T increases the DTD activity by 22-fold but has little or no effect on normal mouse bone marrow cells.[108] Transcription is upregulated and increased levels of DTD, with increased catalytic activity, are observed in the cells 24—48 h after exposure to the inducing agent, oltipraz in this case, but enzyme activity returns to control levels after 72 h in drug free media, although mRNA levels remain elevated.[116] Induction occurs in a dose-dependent manner.[121] The optimal duration of drug exposure is 24 h and more prolonged exposure is associated with lower levels of induction of detoxification enzymes, including DTD. Oltipraz given twice-weekly for 12 weeks did not modulate DTD suggesting that chronic continuous dosing is not useful in induction.[135] These data support intermittent schedules of inducing agents.[116]

An increased DTD level is associated with increased sensitivity to MMC and EO9. Combination therapy with D3T and MMC or E09 produces a 2- or 7-fold enhancement respectively of the cytotoxic effects of these antitumour agents in murine lymphoma cells.[108] This antitumour activity can be inhibited by dicumarol, supporting the proposal that the enhanced tumour cell kill is due to elevated levels of DTD.[130] D3T has been shown to induce DTD activity in 28 of 38 human tumour cell lines, representing 10 tissue types, and significantly increased the cytotoxicity of EO9 in these cells. DTD levels were also induced in normal bone marrow (from low baseline levels), kidney (from low levels) and lung (from intermediate levels) without an increase in toxicity to normal kidney cells.[111] The addition of MMC to D3T did not enhance toxicity in marrow cells whereas D3T with EO9 produced a small increase in marrow toxicity.[108] A further study[113] demonstrated that D3T increased DTD levels (but not the levels of other detoxification enzymes) in all six tumour cell lines studied and this resulted in increased cytotoxicity of MMC in four of the six lines by 1.4 to threefold. In this study, the combination of D3T and MMC was associated with a 50% increase in toxicity in normal human bone marrow cells but this was small in comparison with the enhanced cytotoxicity.[113] Induction of DTD with D3T and ALDs has been shown to reduce the cytotoxicity of benzene and its metabolites on bone marrow stromal cells and lymphocytes.[109,120] The absolute DTD level appears

to be important for induction: DTD activity cannot be induced if there is no baseline DTD activity when cells are homozygous for the NQO1*2 polymorphism[130] and it cannot be further induced if the baseline levels are very high. In the study by Wang et al.[113] the two cell lines that did not exhibit an increase in MMC cytotoxicity had relatively high baseline DTD levels.

Resistance

The clinical role of bioreductive agents may be limited by both inherent and developed drug resistance. MMC resistance may be mediated by increased drug efflux, the repair of DNA lesions or a deficiency in bioreductive enzymes, such as DTD.[136] Down-regulation of DTD is associated with MMC resistance in human gastric, bladder and colon cancer cell lines[29,137–140] and this is not necessarily associated with downregulation of P450 reductase.[29] Resistance by this mechanism can be reversed by transfection of a resistant cell line with DTD and P450 reductase or by placing it under hypoxic conditions.[141] Cell lines often show broad cross-resistance to other cytotoxic agents; this may be due to a "molecular switch" controlling the expression of several detoxification enzymes, some involved in primary resistance and others in cross-resistance. Cisplatin-resistant ovarian cell lines demonstrate increased DTD levels with increased resistance to cisplatin and cross-resistance to MMC.[142] Multidrug resistance involving a decrease in DTD activity has been demonstrated in rat ascites hepatoma cells treated with doxorubicin which were cross-resistant to MMC and the MMC analogue, porfiromycin.[143] Serial exposure of human erytholeukaemic cells to the diaziridinylbenzoquinones, AZQ and BZQ, resulted in resistant cell lines with decreased levels of both DTD and P450 reductase and increased levels of glutathione and superoxide dismutase compared to the parent cell line.[144] These resistant cell lines also demonstrated cross-resistance to doxorubicin and MMC. Resistance to RH1 has been developed in cancer cell lines and studies into the mechanisms of resistance in these lines are ongoing.[145]

Summary

DTD has the potential to be a very important target in oncology. The differential expression of DTD between neoplastic tissue and normal tissue should allow targeting of bioreductive agents to the tumour cells whilst minimising normal tissue toxicity. However, no cell system can predict for the multitude of other factors influencing cytotoxicity and normal tissue toxicity, including tumour heterogeneity, effect of other bioreductive enzymes, degree of hypoxia, cell nutrients, type of damage produced and effectiveness of DNA repair. In addition, none of the bioreductive agents developed to date has been specific for activation by a single reductive enzyme. Previous agents have provided disappointing results, especially EO9 which failed in clinical practice due to a combination of rapid plasma clearance and dose-limiting renal toxicity. Theoretically, RH1 is superior to other bioreductive drugs. It is soluble, has anti-tumour activity in vitro and in vivo and may have a high enough specificity for DTD to allow increased effect in the tumour cells whilst sparing normal tissue. The phase I clinical trial of RH1 in human solid tumours is ongoing. Other areas of interest include manipulation and localisation of DTD and investigation of the mechanisms of drug resistance.

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