Impact of oxaliplatin and a novel DACH-platinum complex in the gene expression of HCT116 colon cancer cells

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Abstract. Novel demethylcantharidin-platinum (DMC-Pt) complexes have been found to have superior *in vitro* anticancer activity against a number of human colon cancer cell lines when compared with oxaliplatin. One complex where the DMC-Pt moiety was integrated with *trans-R,R*-diaminocyclohexane (DACH), exhibited the most pronounced cytotoxicity. To ascertain the mechanistic contribution of the DMC component, microarray analysis was conducted to compare the effect of the novel (*R,R*-DACH)-Pt-(DMC) complex and oxaliplatin, on the gene expression of human colorectal cancer (HCT116) cells. The Affymetrix HG-U133A oligonucleotide microarray was used, and the data allowed for the discrimination of genes that were specifically affected by the DMC ligand. One hundred and forty-one genes were found to be up-regulated. Of these, 48 can be classified according to different cellular responses including DNA repair, DNA synthesis, cell adhesion, cell cycle regulation, mitotic spindle checkpoint and apoptosis/antiapoptosis. The DMC ligand is likely to have caused damage to DNA bases and/or strands, and nucleotide mismatch, as highlighted by the recruitment of the repairing genes from the BER, HR and MMR. Antiapoptotic genes such as *survivin*, *BRCA1* and *ITGB3BP* were up-regulated, and it is proposed that the inherent defense mechanism of the cell may have been triggered, creating potential resistance to apoptosis. This study is the first to demonstrate the impact of the DMC ligand on the gene expression profile of HCT116 colon cancer cells and further substantiates its inclusion in the design of novel platinum-based anticancer complexes.

Introduction

Oxaliplatin is a third generation diaminocyclohexane (DACH)-containing platinum-based antitumour drug that is clinically

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used for the treatment of advanced colorectal cancer and is able to circumvent cisplatin resistance (1). In general, the cytotoxicity of oxaliplatin is comparable to, and occasionally greater than that of cisplatin, but neurotoxicity is a significant side effect (1).

Demethylcantharidin (DMC) (or norcantharidin) is an analogue of cantharidin, an active principle derived from traditional Chinese medicine (*Mylabris*) that has anticancer properties (2). Utilizing a dual mechanism of the drug action approach in designing a novel platinum-based anticancer agent, the complex (*R,R*-DACH)-Pt-(DMC) has been synthesized (3), so that it has the same *R,R*-diaminocyclohexane (DACH)-Pt moiety as oxaliplatin, and DMC as the released ligand (Fig. 1a). We propose that the mechanism of anticancer action is two-fold: i) the *R,R*-DACH-Pt moiety alkylates DNA, and ii) the DMC ligand induces an additional cytotoxic effect in cancer cells. In support of the latter effect, we have demonstrated that (*R,R*-DACH)-Pt-(DMC) generated more DNA lesions when compared with oxaliplatin, and confirmed that the DMC ligand released from (*R,R*-DACH)-Pt-(DMC) participates in DNA damage (4).

DMC has separately been reported to cause loss of cell adhesion (5), inhibition of DNA synthesis (6), cell cycle distortion (3) and apoptosis (3,6,7) (Fig. 1b). Furthermore, cantharidin has been found to be capable of inducing an aberrant mitotic spindle in human lung epithelial carcinoma A549 cells (8).

This study aimed to compare the effect of oxaliplatin and the novel complex (*R,R*-DACH)-Pt-(DMC) on the gene expression of human colorectal cancer (HCT116) cells using microarray technology. The Affymetrix HG-U133A oligonucleotide microarray was used, allowing for the simultaneous examination of a large number of genes by measuring mRNA levels. As the structures of the two test compounds have a common *R,R*-DACH-Pt moiety, the analysis should be able to discriminate the effect due to the DMC ligand. The strategy used for the microarray analysis is shown in Fig. 1b, and the detected genes were sorted according to the observed differences in gene expression levels in cells treated with either (*R,R*-DACH)-Pt-(DMC) or oxaliplatin.

Materials and methods

Chemicals and reagents. Oxaliplatin was supplied by W.C. Heraeus GmbH & Co. KG (Hanau, Germany). DMC was
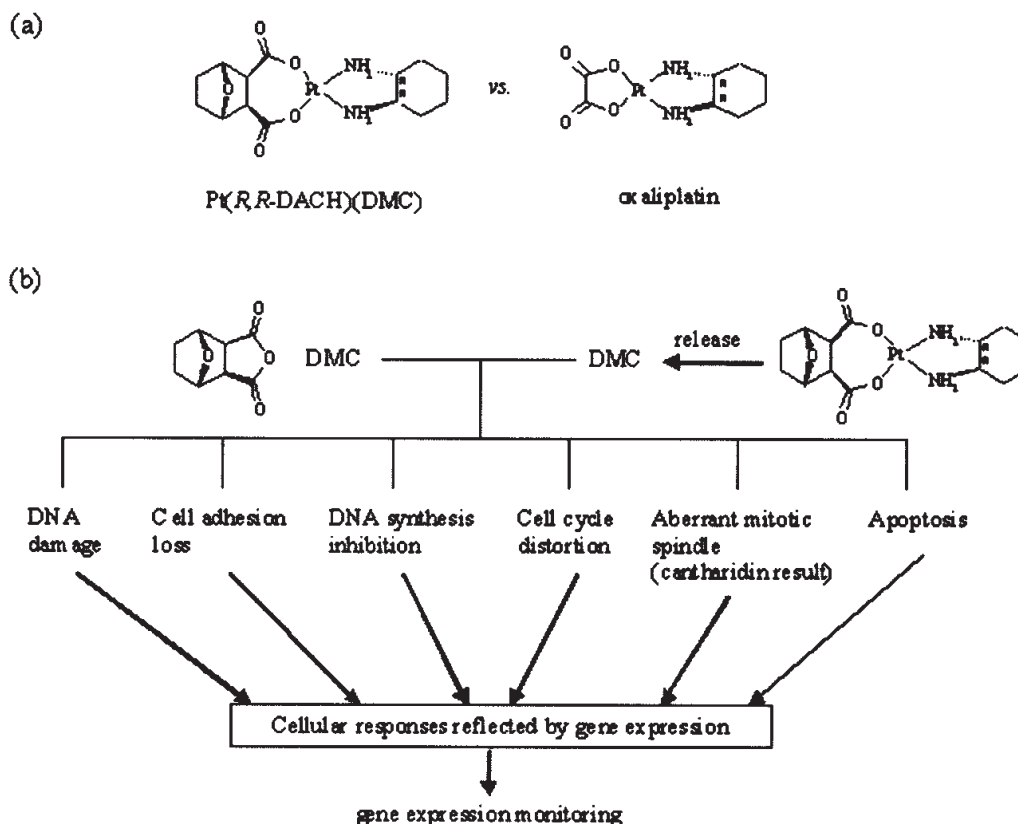
Figure 1. (a) DACH-containing Pt compounds compared in the microarray analysis. (b) Strategy used in the analysis of gene expression changes in response to DMC released from Pt (*R,R*-DACH) (DMC), using the Affymetrix HG-U133A oligonucleotide microarray.

synthesized from the Diels-Alder reaction between furan and maleic anhydride, and (*R,R*-DACH)-Pt-(DMC) was synthesized according to procedures described previously (3).

Cell culture and drug treatment. HCT116 cells from the American Type Culture Collection (ATCC, Rockville, MD, USA) were grown in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μM/ml streptomycin. Cells were plated in tissue culture dishes and incubated (37˚C, 5% CO_2) for 18-24 h before drug treatment. Oxaliplatin and (*R,R*-DACH)-Pt-(DMC) at their IC_{50} values in HCT116 cells (i.e. 1.24 and 0.40 mM, respectively) were exposed to cells for 72 h (3).

Determination of relative gene expression. After drug treatment, adherent cell populations were harvested for subsequent expression profile analysis. Total RNAs were extracted with TriReagent according to the manufacturer's protocol (Molecular Research Center, USA), and purified and cleaned by passing through an RNeasy column (Qiagen, Valencia, USA). The differential expression of genes in HCT116 cells treated with (*R,R*-DACH)-Pt-(DMC) and oxaliplatin was examined by microarray analysis according to the Affymetrix microarray protocol (Affymetrix, Santa Clara, USA). The samples were subjected to oligonucleotide array analysis using the human HG-U133A GeneChip that contains ~54,000 probe sets including 38,500 well-characterized human genes. Cells treated with (*R,R*-DACH)-Pt-(DMC) or oxaliplatin were compared. Experiments were duplicated and

data analyzed with the GeneChip Operating Software (GCOS). The criterion for gene selection was a fold-change of ≥2.0 in the two experiments.

Results

Microarray analysis. The effect on the gene expression of HCT116 cells treated with either oxaliplatin or (*R,R*-DACH)-Pt-(DMC) was compared by microarray technology, where the analysis was able to differentiate genes influenced by the DMC ligand. The results showed that 141 genes were up-regulated and 4 were down-regulated (data not shown). The classification of the function of each gene was according to the information provided by the NetAffx Analysis Center (www.affymetrix.com). Forty-eight genes were classified into different categories based on the cellular responses induced by DMC, and summarized in sub-sections, as shown in Table 1: DNA repair (Table 1a), DNA synthesis (Table 1b), cell adhesion (Table 1c), cell cycle regulation (Table 1d), mitotic spindle checkpoint (Table 1e) and apoptosis/anti-apoptosis (Table 1f). The average fold changes ranged from 2.1 to 3.8. No down-regulated genes were found among the 48 genes. The results from each category will be discussed in detail.

Genes involved in DNA repair. Sixteen genes associated with DNA repair were found to be up-regulated, and a summary of their involvement in the relevant sub-groups is shown in Table 1a. Specific genes included *TOP2A*, which is involved
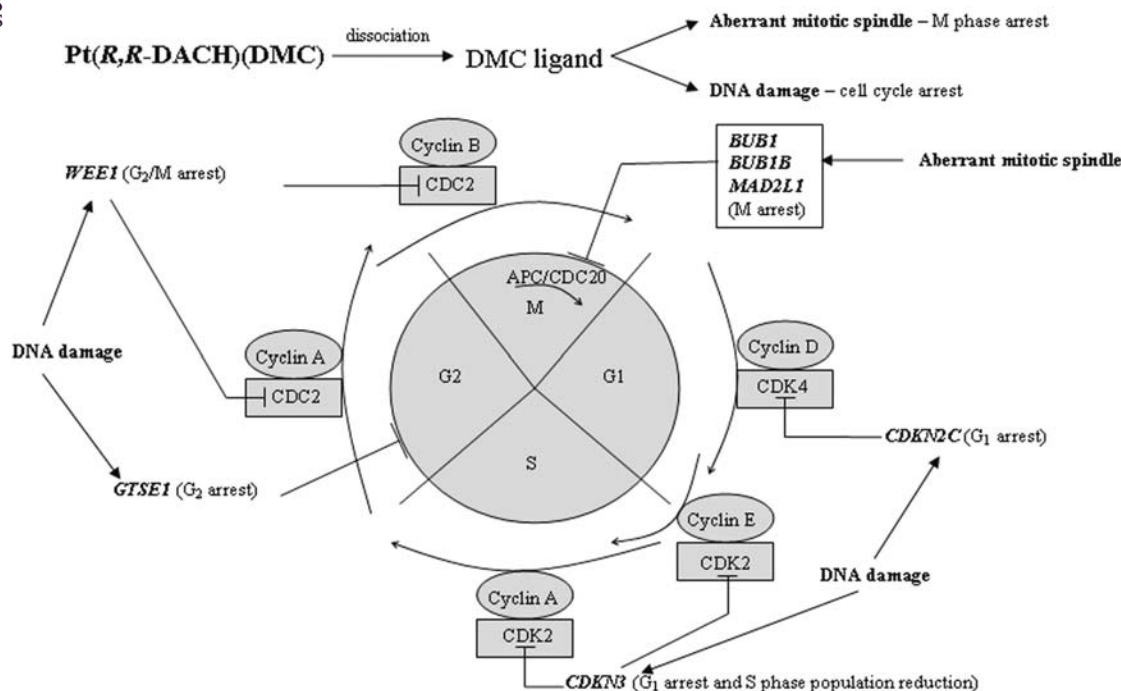
Figure 2. The cell cycle regulation controlled by the expression of cyclin-dependent kinase inhibitor genes induced by the DMC ligand is shown.

in the cleavage and ligation of phosphodiester bonds; *USP1*, which inhibits PCNA activity; *RFC3, 4 and 5*, involved in the loading of PCNA; *RAD51AP1* and *RAD51*, important for strand transfers in homologous recombination (HR); *RAD54B*, which interacts with DNA damage sites in HR; *BRCA1*, a gene that facilitates HR and non-homologous end-joining (NHEJ) processes; *CSPG6*, which contains damaged and undamaged chromatids; *FEN1*, involved in flap cleavages in base excision repair (BER) and the elimination of divergent sequences in HR (9); *POLE2*, involved in DNA synthesis; *HMGB1* and *HMGB2*, important for recognition of the DNA cross-links; and *MSH2* and *MSH*, for recognition of mismatch in mismatch repair (MMR). Among this group of genes, the expression of *HMGB2* was found to be the most affected (average fold change: 3.6).

Genes involved in DNA synthesis. Twenty-eight genes related to DNA synthesis were found to be up-regulated and summarized (Table 1b). Among this group, 8 genes are linked to DNA repair: *TOP2A*, *RFC3*, *4*, and *5*, *FEN1*, *HMGB1*, *HMGB2* and *POLE2*. Seven of these are linked to nucleotide synthesis: *RRM1* and *2*, involved in the production of deoxy-ribonucleotides; *TK1*, which converts thymidine to TMP; *DUT*, which converts dUTP to dUMP; *DCK*, which converts deoxycytidine to dCMP; *PRPS*2, which converts ribose 5-phosphate to 5'-phosphoribosyl-1-pyrophosphate; and *DHFR*, involved in the reduction of folic acid to tetrahydrofolate. Eleven of the 28 genes are related to initiation of DNA replication: *ORC1L*, *MCM2*, *4*, *5*, *6* and *7*, which form the pre-replication complex; *GINS1* and *2*, involved in the unwinding of the replication fork; *PRIM1*, a primer; *CDC6*, involved in the loading of the MCM complex; and *CDC7*, important for the phosphorylation of the MCM complex. From this latter group, MCM4 appears to be the most significant gene as

three probe sets (212141_at, 212142_at and 222036_s_at) were identified, of which one (212141_at) had the highest gene expression (average fold change: 3.6).

Genes involved in cell adhesion. Two genes associated with cell adhesion were up-regulated (Table 1c). *ITGB3BP* activates b3 integrin, whereas *KITLG* promotes cell adhesion directly and activates integrins (10,11). *KITLG* is the more significant gene due to the two probe sets (207029_at and 211124_s_at) being expressed. The average fold change for the 211124_s_at probe set was 2.8.

Genes involved in cell cycle regulation. Eight up-regulated genes involved in cell cycle regulation were detected and summarized (Table 1d and Fig. 2). These include *CDKN2C* which causes G_1 arrest and *CDKN3* which inhibits the G_1/S transition and reduces the S-phase population, *WEE1* which induces G_2/M arrest, and *GTSE1* which causes G_2 arrest and is also a DNA damage response gene. Others include mitotic checkpoint genes *BUB1*, *BUB1B* and *MAD2L1* which cause M-phase arrest. The most prominent gene was *BUB1*, as two probe sets (209642_at and 215509_s_at) were detected where the average gene expression fold change for the 209642_at probe set was the highest at 3.8. The three mitotic checkpoint genes are induced when the mitotic spindle becomes aberrant.

Genes involved in apoptosis and antiapoptosis. The three genes involved in antiapoptosis were: *BIRC5*, *ITGB3BP* and *BRCA1* (Table 1e and Fig. 3). *ITGB3BP* participates in cell adhesion, and *BRCA1* is involved in DNA repair as described previously. The role of *HMGB1* remains unclear as there are reports that it facilitates apoptosis by activating p53 (12), but it has also been suggested that it can act as an antiapoptotic protein (13). The most notable gene was *BIRC5* where two
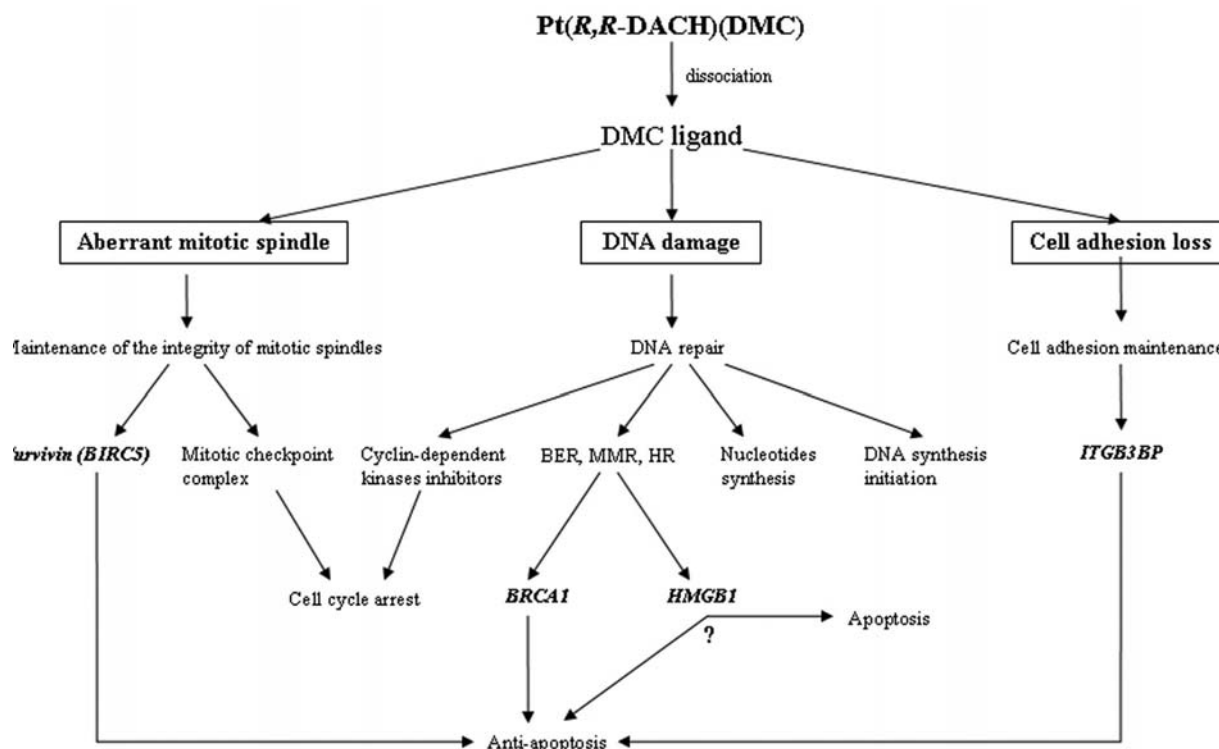
Figure 3. Apoptosis and antiapoptosis genes apparently induced by the DMC ligand.

probe sets (202094_at and 202095_s_at) were observed and the average fold change for the 202095_s_at probe set was the highest (3.6). *BIRC5* is triggered when defects are present in the mitotic spindle assembly.

Discussion

The microarray data revealed that changes in the expression of specific genes can distinguish cellular responses due to the DMC ligand. The results provided further insight into DMC contribution to the overall mechanism of cytotoxicity in HCT116 cells. Selection of the genes was based on their appearance in two experimental trials with a difference in expression of ≥2-fold. Thus, the reliability of the results in this study is significantly increased. The impact of this study is that it has demonstrated that 48 out of 145 genes (one-third of the data) can be correlated with cellular responses induced by DMC (Fig. 1b), as opposed to that of oxaliplatin.

In this study, cells were subjected to drug treatment for 72 h, which was sufficient exposure time to enable the triggering of the DNA repair mechanisms to restore DMC-induced DNA damage. The genes likely to be involved in DNA repair are shown in Table 1a. *POLE2*, *RFC3*, *4 and 5*, and *TOP2A* are recruited in most DNA repair mechanisms, and are found to be overexpressed in cells treated with (*R,R*-DACH)-Pt-(DMC) rather than oxaliplatin. This finding implied that more DNA lesions were formed which required additional repairing units. These observations concurred with our recent report that (*R,R*-DACH)-Pt-(DMC) caused more serious DNA damage when compared with oxaliplatin (4). The results also showed that *FEN1* in BER, *BRCA1*, *RAD51*, *RAD51AP1*, *RAD54B* and *CSPG6* in HR, and *MSH2* and *MSH6* in MMR were overexpressed. Therefore, we conclude

that the DMC ligand can damage DNA bases, induce DNA strand breaks and cause nucleotide mismatch, which are then repaired by the BER, HR and MMR mechanisms, respectively.

NER is generally believed to be involved in the removal of Pt-DNA adducts (14). However, no changes in the expression of genes directly related to NER were found in this analysis. This is consistent with the NCI (National Cancer Institute) microarray data for cantharidin (15). It is noteworthy that the HMGB1 protein, which protects Pt-DNA adducts from recognition by the NER proteins (repair shielding model) and blocks the removal of Pt-DNA adducts (12), and the HMGB1 gene were found to be up-regulated in this study. The implication is that the over-expression of *HMGB1*, induced by the DMC ligand, may assist or enhance the drug action originating from the platinum (Pt-DACH) moiety. However, a recent report showed that introducing a foreign *HMGB1* gene did not influence the cytotoxicity of platinum drugs (16).

The *USP1* gene blocks all DNA repair mechanisms through the deubiquitination of PCNA (17), and its expression in (*R,R*-DACH)-Pt-(DMC)-treated cells was found to be higher than oxaliplatin. Thus, it may be that the DMC ligand is able to disrupt DNA repair.

In this study, GMNN (*Geminin*) was found to be over-expressed in (*R,R*-DACH)-Pt-(DMC)-treated cells. Geminin binds to Cdt1 and prevents its association with the MCM complex, leading to the inhibition of the initiation of DNA synthesis (18). This is supported by a report that DMC inhibited DNA replication, which was tentatively attributed to the cleavage of the Cdc6 protein (6).

Genes facilitating nucleotide synthesis (*RRM1*, *RRM2*, *TK1*, *DUT*, *PRPS2*, *DCK* and *DHFR*), and initiation of DNA replication (*ORC1L*, *CDC6*, *MCM2*, *MCM4*, *MCM5*, *MCM6*,

Table II. Microarray analysis of HCT116 cells treated with Pt (*R,R*-DACH) (DMC) vs. oxaliplatin.[a]

Probe set ID	Gene symbol	Fold change 1	Fold change 2
(a) DNA repair genes			
201291_s_at	*TOP2A*	2.8	3.7
201292_at	*TOP2A*	2.6	2.8
202412_s_at	*USP1*	2.3	2.6
202911_at	*MSH6*	2.0	2.5
203209_at	*RFC5*	2.3	2.6
203210_s_at	*RFC5*	2.3	2.1
204023_at	*RFC4*	2.0	2.3
204127_at	*RFC3*	2.1	3.0
204128_s_at	*RFC3*	2.1	2.1
204146_at	*RAD51AP1*	2.8	3.2
204531_s_at	*BRCA1*	2.3	2.6
204767_s_at	*FEN1*	2.6	2.8
204768_s_at	*FEN1*	2.6	2.5
205024_s_at	*RAD51*	2.1	2.5
205909_at	*POLE2*	2.8	3.5
208808_s_at	*HMGB2*	3.7	3.5
209257_s_at	*CSPG6*	2.3	2.6
209421_at	*MSH2*	2.1	2.1
216508_x_at	*HMGB1* *HMG1L1* *LOC644380*	2.3	2.1
219494_at	*RAD54B*	2.3	2.5
(b) DNA synthesis genes			
48808_at	*DHFR* *LOC643509*	2.1	2.8
201291_s_at	*TOP2A*	2.8	3.7
201292_at	*TOP2A*	2.6	2.8
201477_s_at	*RRM1*	2.3	2.3
201890_at	*RRM2*	2.3	2.8
201930_at	*MCM6*	2.5	2.8
202107_s_at	*MCM2*	2.3	2.3
202532_s_at	*DHFR* *LOC643509*	2.0	3.2
202534_x_at	*DHFR* *LOC643509*	2.3	2.6
202338_at	*TK1*	2.8	2.5
203209_at	*RFC5*	2.3	2.6
203302_at	*DCK*	2.6	2.6
203210_s_at	*RFC5*	2.3	2.1
203401_at	*PRPS2*	2.0	2.5
203967_at	*CDC6*	3.0	2.1
203968_s_at	*CDC6*	2.6	2.5
204023_at	*RFC4*	2.0	2.3
204127_at	*RFC3*	2.1	3.0

Table I. Continued.

Probe set ID	Gene symbol	Fold change 1	Fold change 2
204128_s_at	*RFC3*	2.1	2.1
204510_at	*CDC7*	2.0	3.0
204767_s_at	*FEN1*	2.6	2.8
204768_s_at	*FEN1*	2.6	2.5
205053_at	*PRIM1*	2.1	2.3
205085_at	*ORC1L*	2.3	2.0
205909_at	*POLE2*	2.8	3.5
206102_at	*GINS1*	2.6	2.6
208795_s_at	*MCM7*	2.6	3.0
208808_s_at	*HMGB2*	3.7	3.5
208956_x_at	*DUT*	2.1	2.0
209773_s_at	*RRM2*	2.6	2.5
210983_s_at	*MCM7*	2.5	2.3
212141_at	*MCM4*	4.6	2.6
212142_at	*MCM4*	3.2	2.0
216237_s_at	*MCM5*	3.0	2.5
216508_x_at	*HMGB1* *HMG1L1* *LOC644380*	2.3	2.1
218350_s_at	*GMNN*	2.5	2.6
221521_s_at	*GINS2*	2.6	3.2
222036_s_at	*MCM4*	2.8	2.6
(c) Cell adhesion genes			
205176_s_at	*ITGB3BP*	2.6	2.0
207029_at	*KITLG*	2.6	2.1
211124_s_at	*KITLG*	2.8	2.8
(d) Cell cycle regulation genes			
203362_s_at	*MAD2L1*	3.7	3.2
203755_at	*BUB1B*	3.0	3.7
204159_at	*CDKN2C*	3.2	3.5
204318_s_at	*GTSE1*	2.5	3.0
209642_at	*BUB1*	4.0	3.5
209714_s_at	*CDKN3*	2.3	2.5
212533_at	*WEE1*	2.8	3.0
215509_s_at	*BUB1*	2.5	3.2
(e) Apoptosis and anti-apoptosis genes			
202094_at	*BIRC5*	3.5	2.6
202095_s_at	*BIRC5*	4.0	3.2
204531_s_at	*BRCA1*	2.3	2.6
205176_s_at	*ITGB3BP*	2.6	2.0
216508_x_at	*HMGB1* *HMG1L1* *LOC644380*	2.3	2.1

Table I. Continued.

Probe set ID	Gene symbol	Fold change 1	Fold change 2
(f) Aberrant mitotic spindle response genes			
202094_at	BIRC5	3.5	2.6
202095_s_at	BIRC5	4.0	3.2
203362_s_at	MAD2L1	3.7	3.2
203755_at	BUB1B	3.0	3.7
209642_at	BUB1	4.0	3.5
215509_s_at	BUB1	2.5	3.2

Genes appearing in the two trials with a difference in expression ≥2-fold were considered to be significant in this microarray analysis. [a]Genes are related to (a) DNA repair, (b) DNA synthesis, (c) cell adhesion, (d) cell cycle regulation, (e) apoptosis and antiapoptosis, and (f) aberrant mitotic spindle.

MCM7, CDC7, PRIM1, GINS1 and GINS2) were over-expressed. It is assumed that the up-regulation of these genes is necessary in order to synthesize new strands during the process of repairing DMC-induced DNA lesions (Table 1b). Similarly, overexpression of the DNA replication initiation genes is presumed to be involved in DNA repair with the aim of synthesizing new strands. There is supporting evidence for the correlation of DNA replication initiation with DNA repair, where human Rad51 and Rad52, which participate in HR, have been reported to interact with MCM proteins which are a component of the pre-replicative complex in DNA replication (19). In this regard, RAD51 and MCM2, 4, 5, 6 and 7 were found to be up-regulated in this study.

The microarray data revealed that the expression of ITGB3BP and KITLG was elevated, and it is suggested that this is essential in order to maintain cell adhesion capacity during the slow release of DMC from (R,R-DACH)-Pt-(DMC) (Table 1c).

The progression of a cell cycle is promoted by the activity of cyclin/cyclin-dependent kinase (CDK) complexes, generally regarded as engines driving this process, whereas cell cycle regulation is controlled by cyclin-dependent kinase inhibitors (20). The microarray data indicated that the CDK inhibitor genes such as CDKN2C, CDKN3 and WEE1 were all up-regulated, thus blocking the activities of CDK4, CDK2 and CDC2, respectively (Fig. 2). G_1 arrest should occur as a result of the up-regulation of CDKN2C and CDKN3. A reduction of the S-phase population is likely to be caused by an over-expression of CDKN3, while an overexpression of WEE1 leads to G_2/M arrest. It is reasonable to assume that the release of DMC from the (R,R-DACH)-Pt-(DMC) complex causes more serious DNA damage that results in overexpression of the three cyclin-dependent kinase inhibitor genes, leading to the reinforcement of cell cycle arrest and subsequent DNA repair. The GTSE1 gene, which induces G_2 arrest on DNA damage (21), was also found to be up-regulated. These observations are in agreement with our previous results of the cell cycle distribution where G_1 arrest, S-phase population reduction and G_2/M arrest were clearly demonstrated for

HCT116 cells treated with (R,R-DACH)-Pt-(DMC) or DMC alone (3).

A recent study has indicated that the mitotic spindles were aberrant after cantharidin treatment (8). From this microarray analysis, MAD2L1, BUB1B and BUB1 were highly expressed in cells treated with Pt (R,R-DACH) (DMC). MAD2L1 and BUB1B are components of the mitotic checkpoint complex that cause mitotic arrest when defects are present in the spindle assembly, and in the bipolar attachment of chromosomes (Fig. 2) (22). BUB1 is located on the kinetochores during mitosis, and recruits other mitotic checkpoint proteins (23). An important function of survivin (BIRC5) is to maintain mitotic arrest in response to defects in the mitotic machinery (24), and in this study, expression of BIRC5 was found to be up-regulated. Therefore, it is proposed that DMC can disrupt the organization of the mitotic spindles. As a result, genes related to the mitotic checkpoint complex are up-regulated.

A summary of the apoptotic and antiapoptotic genes apparently induced by the DMC ligand is shown in Fig. 3. Overexpression of several genes [ITGB3BP (25), BRCA1 (26) and BIRC5 (24)] involved in the antiapoptotic process was found. The role of the HMGB1 gene in apoptosis remains unclear as previous reports have indicated that it may facilitate apoptosis by activating p53 (12). However, HMGB1 has been reported as an antiapoptotic protein (13).

In conclusion, the influence of the DMC ligand on the gene expression in HCT116 cells is multi-faceted, and competently demonstrated using microarray technology. For example, the increase in the expression of genes related to DNA repair was triggered by DNA lesions caused by the DMC ligand that is released from the parent complex. The DMC ligand is likely to have caused DNA base damage, DNA strand break, and nucleotide mismatch as exemplified by the BER, HR and MMR genes, respectively, being recruited to repair these lesions. Moreover, the ligand may cause an aberrant mitotic spindle with a subsequent overexpression of genes related to the mitotic checkpoint complex and survivin. Due to further damage induced by the DMC ligand, genes related to the cyclin-dependent kinase inhibitors and the mitotic checkpoint

complex were overexpressed in order to reinforce cell cycle arrest and allow the cells to repair damage. Finally, anti-apoptotic genes were also up-regulated and it is proposed that the inherent defense mechanism of the cell is triggered. These genes may contribute towards resistance against apoptosis which is induced by the DMC ligand, allowing the cells to survive. This study has highlighted the possibility of a dual mechanism of anticancer action exerted by the novel (*R,R*-DACH)-Pt-(DMC) complex, but further studies are required to determine the exact role of DMC.

Acknowledgements

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