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Inhibition of epidermal growth factor receptor over-expressing cancer cells by the aphorphine-type isoquinoline alkaloid, dicentrine

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ABSTRACT

The extraordinary relevance of EGFR in tumour biology makes it an exquisite molecular target for tumour therapy. Despite considerable success with these EGFR tyrosine kinase inhibitors in cancer therapy, resistance against these chemical compounds develops owing to the selection of point-mutated variants of EGFR. Therefore, there is an urgent need for the identification of novel EGFR tyrosine kinase inhibitors for treating tumours with such EGFR mutants. We found a preferential cytotoxicity of dicentrine towards U87MG.ΔEGFR-transduced with a constitutively deletion-activated *EGFR* expression vector as compared to non-transduced wild-type U87MG cells. As determined by microarray-based mRNA expression profiling, this preferential cytotoxicity was accompanied with an activation of BRCA1-mediated DNA damage response, p53 signalling, G1/S and G2/M cell cycle regulation, and aryl hydrocarbon receptor pathways. The activation of these signalling routes might be explained by the fact that dicentrine intercalates DNA and induces DNA strand break by inhibition of DNA topoisomerases. The cell cycle might be arrested by dicentrine-induced DNA lesions.

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1. Introduction

During the past three decades, the members of the epidermal growth factor receptor (EGFR) gene family evolved in cancer biology as important factors affecting prognosis of cancer patients [1–3]. The EGFR family consists of four members (HER1–HER4), which of HER1 (EGFR, *erbB1*) and HER2 (*erbB2*, *c-neu*) are best characterized. They represent a class of glycoproteins, which serve as receptors for EGF and other ligands. They activate signal transduction pathways involved in basic biological processes, such as cell proliferation, apoptosis, metastasis, and angiogenesis. HER1 and HER2 are frequently over-expressed in tumours and are associated with unfavourable prognosis [4].

EGFR molecules are inactive monomers that are activated by binding of specific ligands (*e.g.*, EGF and TGF-α). Activation is associated with either homodimerisation with a second EGFR molecule or with another HER member (*i.e.*, HER2). The dimerisation stimulates the intrinsic tyrosine kinase activity of EGFR, which regulates specific signal transduction cascades. Constitutive EGFR activation as a consequence of mutations or gene amplification causes deregulated cellular processes, such as proliferation, invasion, angiogenesis, cell motility, cell adhesion, inhibition of apoptosis, and DNA synthesis. The kinase activity is also associated with autophosphorylation of five tyrosine residues in the C-terminal EGFR domain. Mutations affecting EGFR expression foster carcinogenesis.

The extraordinary relevance of EGFR in tumour biology makes it an exquisite molecular target for tumour therapy. Apart from therapeutic antibodies, several small molecules have been developed as EGFR inhibitors [5]. For example, gefitinib (Iressa®, Astra Zeneca, DE, USA) and erlotinib (Tarveca®, Genentech Inc., CA, USA) are used for the treatment of non-small cell lung cancer and other tumour types [6]. Both compounds belong to the class of quinazolinamines and exhibit their inhibitory activity on EGFR tyrosine kinases by competing with ATP for the ATP-binding pocket.

Despite considerable success with these EGFR tyrosine kinase inhibitors, resistance develops owing to the selection of point-mutated variants of EGFR [7]. Therefore, there is an urgent need for the identification of novel EGFR tyrosine kinase inhibitors for treating tumours caused by such EGFR mutants.

Dicentrine is an aporphine-type isoquinoline alkaloid isolated from several medicinal plants, some of which have been used in traditional medicines to treat cancers and other diseases [8–15]. Dicentrine has been shown to exert cytotoxic activity towards cancer cells *in vitro* and *in vivo* [16–18]. Dicentrine binds to

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intercalate DNA and inhibits the catalytic activity of DNA topoisomerases [19,20]. Recently, we found that dicentrine binds with high affinity to the erlotinib pharmacophore of EGFR by means of an *in silico* modelling and virtual screening approach [2]. This indicates that EGFR and related signalling pathways may play a role in mediating dicentrine's cytotoxic activity towards cancer cells.

The aim of the present investigation was to investigate, whether dicentrine may exert preferential cytotoxicity towards cancer cells over-expressing EGFR. To this end, we determined the cytotoxicity of dicentrine in non-transduced human U87MG glioblastoma cells and U87MG cells transduced with expression vectors harbouring either wild-type EGFR (U87MG.wtEGFR), deletion-activated EGFR (U87MG.ΔEGFR), or EGFR with a point mutation in the tyrosine kinase domain (U87MG.PK). Furthermore, transcriptome-wide mRNA expression profiling of these cell lines with and without dicentrine treatment has been performed to elucidate signalling pathways involved in the cytotoxicity of dicentrine in transduced and non-transduced cell lines. Interestingly, a connection of BRCA1-mediated DNA damage response and other DNA damage response pathways has been unravelled, indicating a relationship of EGFR and DNA damage signalling upon challenge of cancer cells with dicentrine.

2. Material and methods

2.1. Drugs

Dicentrine was kindly provided by the Drug Synthesis and Chemistry Branch, Chemotherapeutic Agents Repository, National Cancer Institute, USA. The chemical structure of dicentrine is shown in Supplementary Fig. 1.

2.2. Cell lines

The establishment of the parental human glioblastoma cell line, U87MG and its derivatives, which over-express exogenous wild-type EGFR (U87MG.wtEGFR-2N), tyrosine kinase-deficient EGFR (U87MG.DK-2N), or constitutively active EGFR with a genomic deletion of exons 2–7 (U87MG.ΔEGFR) has been described elsewhere [21]. The cell lines were kindly provided by Dr. W.K. Cavenee (Ludwig Institute for Cancer Research, San Diego, CA, USA). Cell culture conditions of these cell lines were as described [22].

2.3. XTT proliferation assay

The toxicity of compounds was determined by means of the Cell Proliferation Kit II (Roche Diagnostics, Mannheim, Germany). This test is based on the cleavage of the yellow 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt (XTT) by ubiquitous dehydrogenases leading to the formation of an orange formazan dye [23]. The amount of dye is commensurate to the number of metabolic active cells. Fresh stock solutions of each compound were prepared in DMSO at a concentration of 100 mM. A dilution series ranging from 10^{-9} M to 10^{-3} M was prepared using DMEM medium to perform the XTT test. Cells were diluted to a final concentration of 1×10^5 cells/mL. One hundred micro-litres of the cell suspension were sowed into the wells of a 96-well culture plate (Costar, Corning, USA). Marginal wells were filled with 100 μL of pure medium, in order to minimize effects of evaporation. Besides, wells filled with medium were required to determine the background absorbance caused by non-metabolized XTT. A row of wells containing cells was left untreated, and another row of wells containing cells was treated with 1 μL DMSO. This served as solvent control. The other rows of wells containing cells were supplemented with different concentrations of compound. Each concentration was tested in at least two independent plates containing different batches of cells.

After incubation with compounds at 37 °C, 5% CO_2 in humidified atmosphere, XTT reagent was freshly prepared and added to each well as specified by the manufacturer: XTT-labelling reagent and electron-coupling reagent were mixed in a ratio of 50:1, and 50 μL of this mixture were added to each well of the 96-well plate. The plates were incubated for about 3 h at 37 °C, 5% CO_2 in humidified atmosphere and read out after incubation. Quantification of cell cytotoxicity was performed in an ELISA plate reader (Bio-Rad, München, Germany) at 490 nm with a reference wavelength of 655 nm. Absorbance values at both wavelengths were subtracted. The cytotoxic effect of the treatment was determined as percentage of viability and compared to untreated cells [24]. The toxicity of compounds was determined by means of the formula:

$$\text{cell viability}[\%] = \frac{\text{absorbance of sample cells}}{\text{absorbance of untreated cells}} \times 100$$

Implemented in the simple ligand binding module of Sigma plot software (version 10.0).

2.4. RNA isolation

Total RNA of EGFR-transduced and non-transduced U87MG human glioblastoma cells was extracted from the test samples using RNeasy® Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions to obtain highly pure RNA. Isolated total RNA was re-suspended in sample buffer provided by the manufacturer. The concentration and quality of total RNA were verified by electrophoresis using the total RNA Nanochip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Berlin, Germany). Only samples with RNA index values greater than 8.5 were selected for expression profiling. RNA concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). All of the RNA samples were stored at −80 °C until used for microarray analyses.

2.5. Probe labelling and Illumina Sentrix BeadChip array hybridisation

Biotin-labelled cRNA samples for hybridisation on Illumina Mouse Sentrix-8 BeadChip arrays (Illumina Inc., San Diego, CA, USA) were prepared according to Illumina's recommended sample labelling procedure based on a previously published protocol [24,25]. In brief, 250 ng total RNA were used for complementary DNA (cDNA) synthesis, followed by an amplification/labelling step (*in vitro* transcription) to synthesize biotin-labelled cRNA according to the MessageAmpII aRNA Amplification kit (Ambion, Inc., Austin, TX). Biotin-16-UTP was purchased from Roche Applied Science, Penzberg, Germany. The cRNA was column purified according to TotalPrep RNA Amplification Kit, and eluted in 60 μL of water. Quality of cRNA was controlled using the RNA NanoChip Assay on an Agilent 2100 Bioanalyzer and spectrophotometrically quantified (NanoDrop).

Hybridisation was performed at 58 °C, in GEX-HCB buffer (Illumina Inc.) at a concentration of 50 ng cRNA/μL, unsealed in a wet chamber for 20 h. Spike-in controls for low, medium and highly abundant RNAs were added, as well as mismatch control and biotinylation control oligonucleotides. Microarrays were washed twice in E1BC buffer (Illumina Inc.) at room temperature for 5 min. After blocking for 5 min in 4 mL of 1% (w/v) blocker casein in phosphate buffered saline Hammarsten grade (Pierce Biotechnology Inc., Rockford, IL), array signals are developed by a 10 min incubation in 2 mL of 1 μg/mL Cy3-streptavidin

(Amersham Biosciences, Buckinghamshire, UK) solution and 1% blocking solution. After a final wash in E1BC, the arrays are dried and scanned.

2.6. Scanning and data analysis

Microarray scanning was done using a bead station array scanner, setting adjusted to a scaling factor of 1 and PMT settings at 430. Data extraction was done for all beads individually, and outliers are removed when >2.5 MAD (median absolute deviation). All remaining data points were used for the calculation of the mean average signal for a given probe, and standard deviation for each probe was calculated.

Data analysis was done by normalisation of the signals using the cubic spline algorithm after background subtraction, and differentially regulated genes are defined by calculating the standard deviation differences of a given probe in a one-by-one comparison of samples or groups. Pathway analysis was done by using the Ingenuity Pathways Analysis software (version 5.5) from Ingenuity Systems (Redwood City, CA, USA).

2.7. Real-time RT-PCR

The experiment was done on the Roche LC480 using ABgene Mastermix "CM 215-A" and Roche Universal Probe Library. Oligos were designed using Roche ProbeFinder Webservice (http://qpcr2.probefinder.com/organism.jsp) and were synthesised by MWG. Two versions were designed per gene. All variants analysed span an intron to prevent cross-reactions with possible genomic DNA contaminations.

Total reaction volume was 10 μL. We used 400 nM final oligo concentration each, 100 nM final probe concentration and 6.3 ng RNA (transcribed in cDNA using Superscript) per reaction. We used transcribed cDNA from RNA obtained from untreated cells or cells treated with dicentrine at the 50% inhibition dose (IC_{50}). No DNAse digest was done. Genes identified by microarray analyses were exemplarily analysed for validation by means of real-time RT-PCR (*CDKN1A*, *E2F1*). The housekeeping genes, *HPRT1*, *G6PD*, *ALAS1*, and *HMBS*, served as reference. The primer sequences are shown in Table 1.

All measurements are done in triplicates to calculate mean values and standard deviations. To calculate normalised mRNA expression values, we measured the sample's crossing point (expressed as a cycle number), the efficiency of the reaction, and the number of cycles completed to determine how much the DNA concentration must have increased for each sample by the end of the amplification. The analysis used these calculations to compare samples and to generate ratios as indicated by the manufacturer (Roche, Penzberg, Germany). The final ratio resulting from the calibrator-normalised relative quantification was a function of PCR efficiency and of the determined crossing points. The

Table 1
IC_{50} values for dicentrine of different U87MG cell lines and respective degrees of sensitisation to dicentrine of transduced cell lines in comparison to non-transduced cells. The IC_{50} values were calculated from the dose–response curves shown in Fig. 1.

Cell line	IC_{50} value (μM)	Degree of sensitization
U87MG	43.0 ± 4.44[a]	–
U87MG.ΔEGFR	4.73 ± 0.29	9.09[b]
U87MG.DK-2N	8.69 ± 0.09	4.95
U87MG.wtEGFR-2N	10.4 ± 0.48	4.13

[a] Mean SEM of two independent experiments with each fourfold determination.
[b] IC_{50} of the corresponding cell line divided by IC_{50} of U87MG.

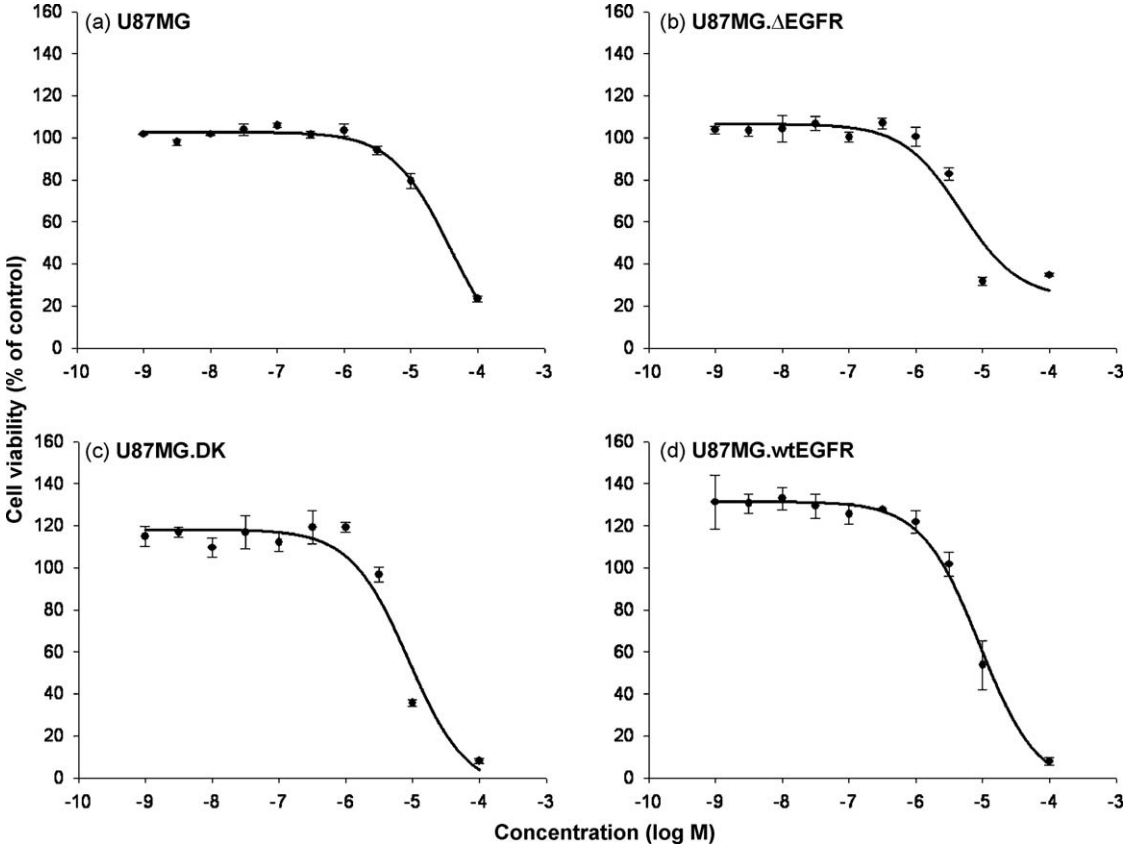

Fig. 1. Cytotoxicity towards dicentrine towards transduced and non-transduced U87MG cell lines as determined by the XTT assay. Dose–response curves of two independent experiments are shown (mean values and SEM of each four measurements).

"concentration ratio" was determined as ratio of the target and the reference genes in the specific sample. To calculate the change of expression between untreated (calibrator) and dicentrine-treated samples, the concentration ratios of the sample were divided by the concentration ratio of the calibrator. This value is the "normalised ratio".

3. Results

3.1. Cytotoxicity of dicentrine

Transduced and non-transduced U87MG glioblastoma cell lines were treated with different concentrations of dicentrine and subjected to the XTT assay. The dose–response curves are shown in Fig. 1 and the 50% inhibition concentrations (IC_{50}) calculated from the dose–response curves in Table 1. The IC_{50} value for dicentrine was 4.13–9.09-fold higher in non-transduced U87MG cells (43.0 ± 4.44 μM) as compared to the three transduced cell lines. U87MG.ΔEGFR cells transduced with a deletion-activated *EGFR* expression vector were most sensitive towards dicentrine (4.73 ± 0.294 μM). The IC_{50} value of U87MG.DK-2N cells carrying a point mutation in the tyrosine kinase domain of EGFR was 8.69 ± 0.09 μM, and the IC_{50} for U87MG.wtEGFR-2N was 10.4 ± 0.48 μM.

3.2. Differential gene regulation upon dicentrine treatment

Next, we were interested, whether specific mRNA expression profiles underlie the different IC_{50} values. Therefore, we performed microarray-based mRNA hybridisations. An overview of the number of up- or down-regulated genes upon treatment of the different U87MG cell lines with dicentrine is given in Fig. 2 and Supplementary Table 1. While the majority of differentially regulated genes were different between the different cell lines, 8 genes were commonly regulated in U87MG and U87MG.wtEGFR-2N cells, 10 genes in U87MG and U87MG.ΔEGFR, and 37 genes in U87MG and U87MG.DK-2N cells.

The non-intersected or the uniquely regulated genes for dicentrine were considered as those molecular players that are responsible as mechanism of action for this compound. The variability in the number of such uniquely regulated genes in different EGFR-transduced cell lines suggested the diverse pathways activated by individual compounds to elicit their cellular effects.

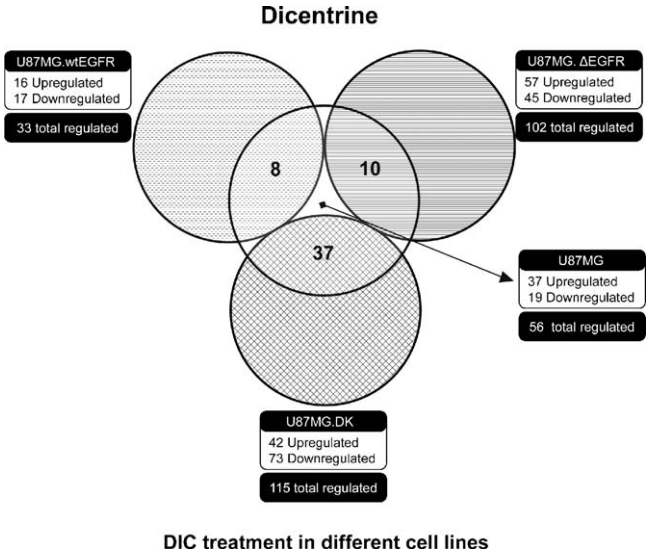

Fig. 2. Genes uniquely and commonly regulated in transduced and non-transduced U87MG cell lines. Venn diagram representation was chosen to compare overlaps of differentially expressed genes after dicentrine treatment.

In order to see, whether dicentrine elicits its activity through one or several pathways, we investigated which signalling pathways were affected by the action of dicentrine in U87MG.ΔEGFR cells. For this reason, we analyzed all genes included in the Illumina biochip by means of the Ingenuity Pathway Analysis software. Out of >80 canonical pathways, 5 were significantly regulated upon dicentrine treatment ($P < 0.05$). The 10 functional groups of genes with the lowest *P*-values are shown in Fig. 3. Of them, genes involved in BRCA1-mediated DNA damage response were the most prominent. Based on this result, we then asked which genes were affected upon dicentrine treatment. The genes associated with BRCA1-mediated DNA damage response are shown in Fig. 4.

In addition to BRCA1-mediated DNA damage response, other pathways, which appeared in dicentrine-treated U87MG.ΔEGFR but not in non-transduced U87MG cells, were the aryl hydrocarbon receptor (AHR) signalling route, the G1/S as well as the G2/M cell cycle checkpoint regulation routes, and the p53 signalling pathway (Fig. 3). All of these pathways are related to DNA damage.

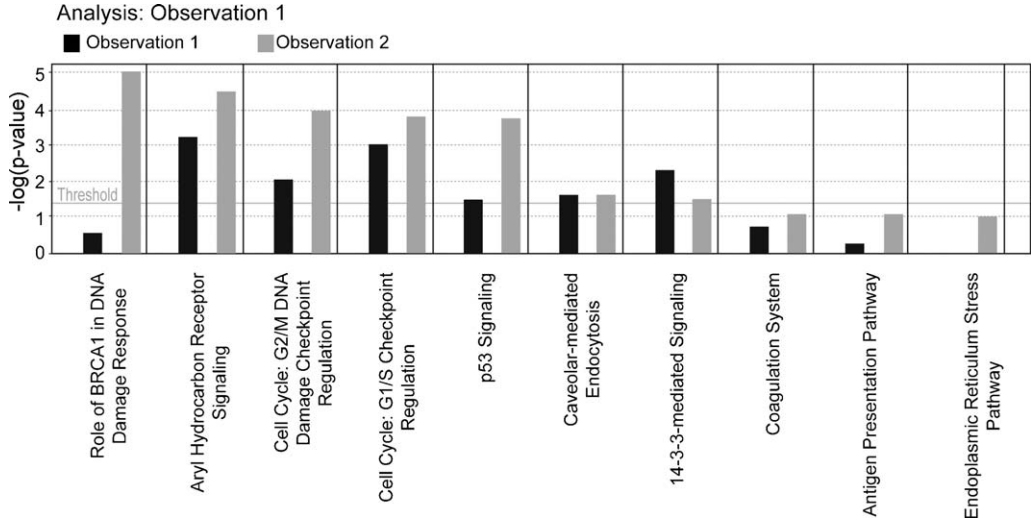

Fig. 3. Comparison of canonical pathway analysis for dicentrine-treated non-transduced U87MG and U87MG.ΔEGFR-transduced with a deletion-activated EGFR expression vector. Each bar represents the ratio of the number of genes that are regulated in a particular pathway upon treatment with dicentrine (IC_{50}) for 72 h. Only the top 10 canonical pathways are shown of U87MG (dark grey bars) and U87MG.ΔEGFR (light grey bars) as analyzed by the Ingenuity Pathway Analysis software.

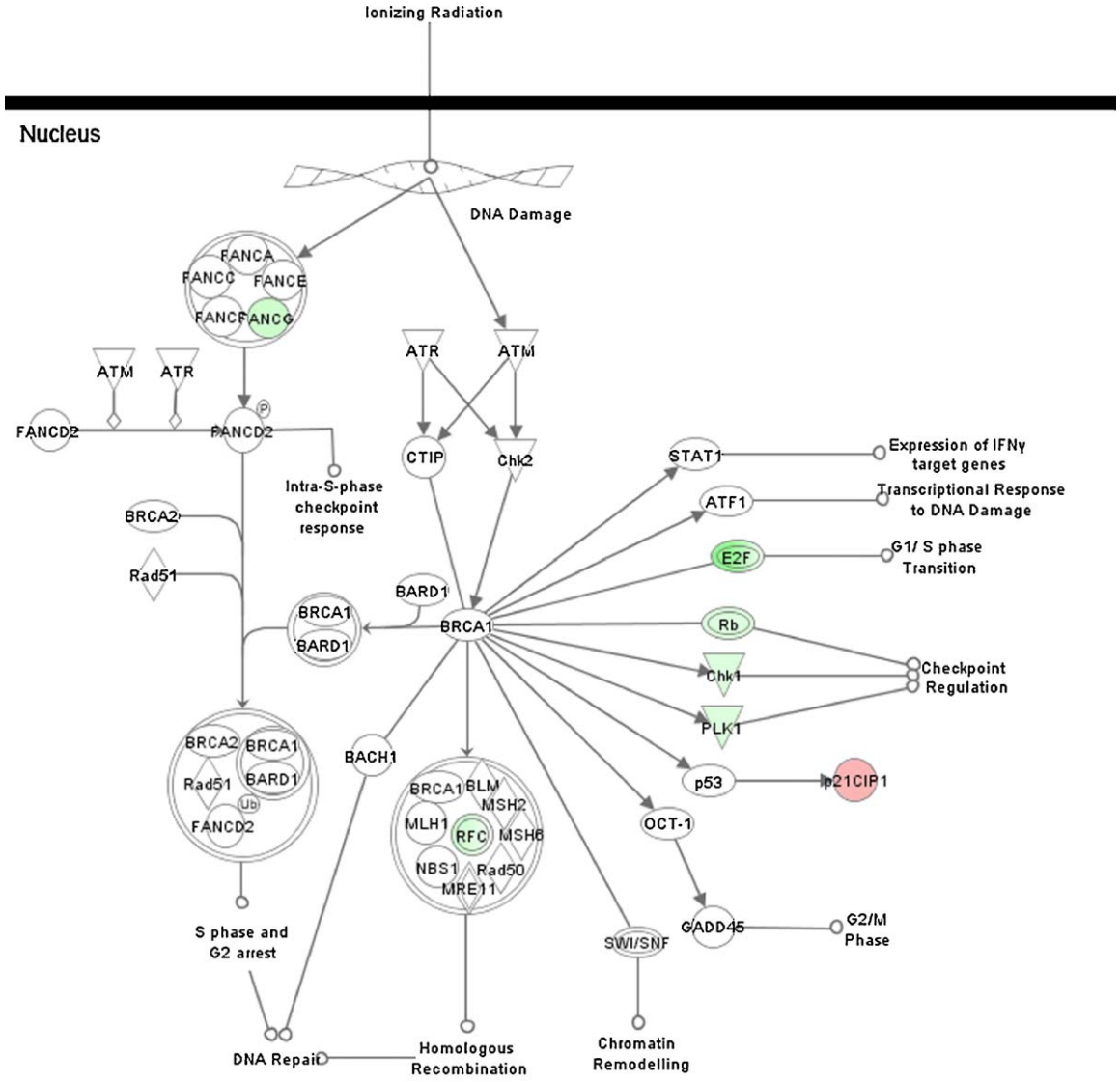

Fig. 4. BRCA1-mediated DNA damage response pathway. This pathway was identified as top canonical pathway differentially regulated by dicentrine in U87MG and U87MG.ΔEGFR cells. Genes in red colour indicate up-regulation, whereas genes in green colour indicate down-regulation of mRNA expression upon dicentrine treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.3. Validation of microarray data by real-time RT-PCR

The results of DNA microarray hybridisations were exemplarily validated by real-time RT-PCR. For this purpose, two genes were chosen. One gene (*CDKN1A*) was found to be up-regulated, another one (*E2F1*) was down-regulated upon dicentrine treatment in microarray analyses. As can be seen in Table 2, corresponding up- or down-regulation of these

Table 2
Validation of mRNA microarray results.

Gene	Cell line	Method	Untreated cells	Dicentrine-treated cells	Fold change
CDKN1A[a]					
	U87MG	Microarray	10.604.6 ± 310.6[b]	20.263.1 ± 596.2	1.7
		RT-PCR	0.137 ± 0.0832[c]	0.533 ± 0.0465	3.89
	U87MG.ΔEGFR	Microarray	7.846.0 ± 197.1	21.471.0 ± 472.6	2.7
		RT-PCR	0.025 ± 0.0013	0.268 ± 0.0094	10.74
E2F1[d]					
	U87MG	Microarray	330.0 ± 14.8	150.0 ± 5.1	−2.2
		RT-PCR	0.003 ± 0.0012	0.001 ± 0.0004	−3.21
	U87MG.ΔEGFR	Microarray	505.3 ± 18.1	135.2 ± 5.8	−3.7
		RT-PCR	0.001 ± 0.0001	0.000 ± 0.000	−3.77

Genes were selected for real-time RT-PCR analysis from the list of differentially expressed genes.
[a] *Homo sapiens* cyclin-dependent kinase inhibitor 1a (p21, Cip1) (*CDKN1A*), transcript variant 2.
[b] Average hybridisation signal (±SEM).
[c] Conc. ratio (±Conc. ratio STD).
[d] *Homo sapiens* E2F transcription factor (E2F1).

Table 3
Relationship between cell doubling times and microarray-based mRNA expression of cell cycle-, proliferation- or apoptosis-related genes to dicentrine in tumour cell lines of the N.C.I. drug screening panel (http://dtp.nci.nih.gov).

Symbol	Name	*P*-value (Fisher exact test)
Cell cyle and proliferation		
–	Cell doubling time	0.0002
CCNE1	Cyclin E1	0.017
CCNF	Cyclin F	0.016
PAK4	P21(CDKN1A)-activated kinase 4	0.039
VEGFB	Vascular endothelial growth factor B	0.011
Apoptosis		
CASP-7	Apoptosis-related cysteine peptidase	0.004
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.008
IKB	Inhibitor of NFKB	0.069
PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)	0.007
DAPK1	Death-associated protein kinase 1	0.040

genes after dicentrine treatment was also observed by real-time RT-PCR.

3.4. Cell cycle and apoptosis parameters

To analyze the role of cell cycle and apoptosis for response of tumour cells toward dicentrine in a general context, we correlated the IC_{50} values for dicentrine of the tumour cell line panel of the Developmental Therapeutics Program of the National Cancer Institute, USA, with the cell doubling times of the cell lines and the microarray-based mRNA expression of genes involved in cell cycle and apoptosis (http://dtp.nci.nih.gov). As shown in Table 3, the IC_{50} values for dicentrine significantly correlated with the cell doubling times of the cell lines. The mRNA expression of *CCNE1* and *CCNF* and the proliferation-related genes, *PAK4* and *VEGFB* were also significantly associated with the IC_{50} values for dicentrine. Other cyclins did not correlate with dicentrine.

Furthermore, we analyzed apoptosis-related genes and found significant relationships between the expression of *CASP-7*, *NFKB1*, *PDCD4*, and *DAPK1* to the IC_{50} values for dicentrine (Table 3). An inverse correlation with borderline significance ($P = 0.07$) was observed for the NFκB-inhibitor, *IKB*. Other caspases as well as bcl-2 family members (bcl-2, bax, bcl-x, bxl-xL, and bad) did not correlate with response of tumour cell lines towards dicentrine.

4. Discussion

In the present investigation, we found a preferential cytotoxicity of dicentrine towards U87MG.ΔEGFR-transduced with a constitutively deletion-activated *EGFR* expression vector as compared to non-transduced wild-type U87MG cells. A possible explanation for preferential cytotoxicity of dicentrine towards EGFR-over-expressing cells is that dicentrine binds to EGFR receptor. Hence, EGFR-expressing cells are killed, while non-EGFR-expressing cells survive. A mean energy of docking of −8.1 kcal/mole and a p*K*i of 4.25 nM have been calculated *in silico* by 3D molecular docking of dicentrine to the three-dimensional structure of the binding site of the EGFR tyrosine kinase, erlotinib, at the ATP-binding site of EGFR [2]. Based on this result, we have started the present investigation. The preferential cytotoxicity of dicentrine in EGFR-transfected cells found by XTT assays support the view that dicentrine inhibits EGFR-expressing cells by binding to EGFR.

The preferential activity of dicentrine towards U87MG.ΔEGFR cells was indeed EGFR-specific, since we and others found that this cell line exhibited resistance rather than sensitivity towards other DNA damaging agents such as cisplatin or artesunate [26,27]. Anti-cancer with other modes of action (paclitaxel, vincristine, homoharringtonine, L-alanosine) was also less active in U87MG.ΔEGFR cells [28,29]. Comparable data have been resported by other authors in other EGFR-expressing cells [30].

This preferential cytotoxicity was accompanied with an activation of BRCA1-mediated DNA damage response, p53 signalling, G1/S and G2/M cell cycle regulation, and aryl hydrocarbon receptor pathways. The activation of these signalling routes might be explained by the fact that dicentrine intercalates DNA and induces DNA strand break by inhibition of DNA topoisomerases. The cell cycle might be arrested by dicentrine-induced DNA lesions.

Indeed, an association of dicentrine with DNA damage has previously been reported. Dicentrine intercalates DNA and inhibits DNA topoisomerases I and II [19,31]. In this respect, dicentrine may be compared with classical anti-cancer drugs such as anthracyclines. The interrelationship of EGFR and BCRA1 has not well been investigated [32].

There are hints that EGFR inhibits the catalytic and DNA-cleaving activity of DNA topoisomerase II [33,34]. Furthermore, inhibition of EGFR by small molecule inhibitors such as gefitinib resulted in synergistic interaction with the DNA topoisomerase inhibitor, irinotecan [35]. The sensitivity of BRCA1- and BRCA2-deficient etoposide-induced DNA double strand breaks was mediated by DNA topoisomerase II [36], indicating a mechanistic relationship between both proteins. Although incompletely understood at present, it is worth speculating that EGFR cross-talks with DNA toposiomerases and BRCA1-mediated DNA damage response pathways. This cross-talk may be important for intercalation and DNA topoisomerase-mediated DNA damage of dicentrine.

Apart from the BRCA1-mediated DNA damage response pathway, other signalling routes were also differentially regulated between U87MG.ΔEGFR and parental U87MG cells. Among them were two signalling routes for arrest in G1/S or G2/M cell cycle phases. There is a plethora of investigations pointing to the eminent role of cell cycle arrest for cancer therapy. Cancer cells arresting in one of these phases can resist the detrimental effects of cancer chemotherapy [37,38]. On the other side, compounds capable to release arrested cells such as caffeine lead to a re-sensitization of cancer cells towards chemotherapy [39]. The fact that dicentrine differentially affects signalling routes for G1/S or G2/M arrest in U87MG and U87MG.ΔEGFR cells indicates that dicentrine interferes with cell cycle of cancer cells, possibly via the induction of DNA lesions.

The p53 signalling pathway was also activated in U87MG.ΔEGFR cells upon challenge with dicentrine. It is well-known that p53 is activated by DNA lesions, either spontaneously occurring due to the tumour suppressor function of p53 or upon chemotherapeutic treatment. In both cases, p53 can mediate cell cycle arrest, DNA repair, and/or apoptosis. We conclude that the results obtained by microarray hybridisations may point to dicentrine-induced DNA lesions, as already been found by others [19,31,40].

This point of view is also conceivable with the finding that the aryl hydrocarbon receptor (AhR) signalling pathway was involved in the action of dicentrine. This signalling pathway is of enormous relevance in carcinogenesis, but also for resistance towards chemotherapy. Induction of the *mdr1* gene, which is well-known to induce multidrug resistance, requires the AhR nuclear translocator [41]. Down-regulation of AhR repressor conferred resistance

to apoptotic signals [42] and 17-β-estradiol-induced AhR over-expression with the characteristic phenotype of increased proliferation and resistance to apoptosis [43]. Given the general role of AhR for drug resistance, it is worth hypothesising that AhR may also be involved in response of tumour cells to dicentrine, as indicated by the results of the present investigation.

CDKN1A (p21, Cip1) appeared as one of the differentially expressed genes upon dicentrine treatment in our microarray experiments. This gene encodes a cyclin-dependent kinase inhibitor, which blocks cell cycle progression at the G1 phase by interacting with cyclin E. Interestingly, *CCNE1* (cyclin E1) was one of the genes which significantly correlated with response of the N.C.I. cell line panel to dicentrine. This indicates that cell cycle progression may be inhibited by CDKN1A-cyclin E interaction upon dicentrine-induced DNA damage.

It should be taken into account that dicentrine as many other natural compounds alike targets many cellular structures in the organism and is not cancer-specific in its bioactivity spectrum. Besides its cytotoxic activity, dicentrine inhibited trypanosomes [15]. Dicentrine is a known inhibitor of α-adrenoreceptors [44–47]. It reduced hypertension and hyperlipidemia, two of the major risk factors for cardiovascular disease [48]. Electrophysiological examination of dicentrine on the conduction system of rabbit hearts showed that dicentrine exerted anti-arrhythmic action [49]. Dicentrine had beneficial effects on the rigidity of head and body circulation. It improved tube distensibility and wave transmission time [50]. Furthermore, dicentrine inhibited sodium and potassium channels [51] and thromboxane formation during platelet aggregation [52]. It also exerted chromosomal damaging clastrogenic effects [40].

If dicentrine will be further developed for cancer treatment, derivatives with preferential activity towards cancer cells and fewer side effects on other tissues and cellular targets should be generated. Dicentrine represents an example of a natural product with multi-target activity. It is valuable as lead compound for the derivatisation of second generation compounds with improved pharmacological features.

In conclusion, the data of the present investigation imply that otherwise drug-resistant tumours with constitutive activation and/or over-expression of EGFR may be preferentially susceptible towards dicentrine due to inhibition of EGFR and regulation of EGFR-related downstream signalling pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.11.025.

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