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Drosophila melanogaster deoxyribonucleoside kinase activates gemcitabine

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ABSTRACT

Drosophila melanogaster multisubstrate deoxyribonucleoside kinase (Dm-dNK) can additionally sensitize human cancer cell lines towards the anti-cancer drug gemcitabine. We show that this property is based on the Dm-dNK ability to efficiently phosphorylate gemcitabine. The 2.2 Å resolution structure of Dm-dNK in complex with gemcitabine shows that the residues Tyr70 and Arg105 play a crucial role in the firm positioning of gemcitabine by extra interactions made by the fluoride atoms. This explains why gemcitabine is a good substrate for Dm-dNK.

Introduction

Mammals have four different deoxyribonucleoside kinases (dNKs) [1]. There are two cytosolic dNKs, thymidine kinase 1 (TK1, EC 2.7.1.21) and deoxycytidine kinase (dCK, EC 2.7.1.74), and two mitochondrial dNKs, deoxyguanosine kinase (dGK, EC 2.7.1.113) and thymidine kinase 2 (TK2, EC 2.7.1.21) with distinct but overlapping specificities [2–4]. On the other hand, *Drosophila melanogaster* has only one kinase, multisubstrate dNK (Dm-dNK), which combines a wide substrate acceptance with the exceptionally high turnover rates. Dm-dNK substrate specificity can be easily manipulated, either by random mutagenesis or by knowledge based protein engineering [5–7].

Several dNKs play a key role in the chemotherapeutic treatment of cancer and viral diseases as they catalyze the first, and often rate-limiting step of the nucleoside analogs (NA) activation [1]. These enzymes have therefore been preferred candidates to be used as suicide genes in gene-therapy of cancer. The *Herpes* simplex virus 1 thymidine kinase (HSV1-TK) has served as the prototype for suicide gene-therapy. The introduction of HSV1-TK as a suicide gene into tumor cells is followed by addition of a NA, e.g. ganciclovir (GCV). The HSV1-TK activates GCV and traps it inside the cell by phosphorylation, hence being the first and rate limiting step towards a toxic effect of the NA inside the cell [8,9]. Dm-dNK, when expressed in human cancer cell lines, has been shown to increase the sensitivity of the cell towards several cytotoxic NAs [10].

Gemcitabine has lately been described as one of the most promising new cytotoxic agents in cancer therapy, and it has shown activity in a variety of hematological malignancies and solid tumors. It is one of the most active drugs against non-small lung cancer, pancreatic, bladder, ovarian and breast cancer. Human dCK is the main activator of gemcitabine and since this NA has a low toxicity profile, it is a prime candidate for suicide gene-mediated gene-therapy of cancer [11,12].

We show in this paper that Dm-dNK was able to sensitize two human cancer cell lines towards the pyrimidine analog gemcitabine. We also determined the structure of wild type Dm-dNK complexed with gemcitabine and were thereby able to explain the structural basis for the interactions of Dm-dNK with gemcitabine.

Materials and methods

Materials. ^{3}H labeled gemcitabine (2′-deoxy-2′,2′-difluorocytidine, [cytosine-5-3H]-) was obtained from Moravek Biochemicals Inc., Brea, CA. Unlabeled gemcitabine was from Thykn International (Mumbai, India).

Construction of a retrovirus vector expressing Dm-dNK. The generation of the vector, a retrovirus based on the Moloney murine

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leukemia virus (pLCXSN) containing Dm-dNK under the control of the cytomegalovirus promoter, has been described by Knecht et al. [7].

Cell lines and retroviral transduction. The human cancer cell line brain glioblastoma U-87-MG (ATCC HTB-14) was purchased at American Type Culture Collection (ATCC). The dCK deficient ovarian cancer cell line AG6000 was a kind gift from Dr. G.J. Peters (Dept. Oncology, VU University Medical center, Amsterdam). Cells were cultured in RPMI, E-MEM, Leibovitz's L-15 and D-MEM medium, respectively; with 10 % (v/v) Australian originated fetal calf serum and 1 ml/l of gentamicin. Cells were grown at 37 °C in a humidified incubator with a gas phase of 5% CO_2. The cells were transduced with the recombinant retrovirus with 5 MOI in fully conditioned medium mixed with 5 μg/ml of Polybrene, incubated for 48 h and then cultured continuously for 3 weeks in the presence of 300–400 μg/ml Genetecin® (Life Technologies Inc.).

Cell proliferation assay – Cytotoxicity. U-87-MG and AG6000 cells were plated at density ranges of 1500–3500 cells/well, dependent on cell line growth, in 96-well plates coated with poly-L-lysine (Sigma). The different analogs of interest were suspended in conditioned media and added after 24 h of incubation, 200 μl/well. Cell survival was assayed after 96–120 h of drug exposure by XTT cell proliferation kit (Roche). The data (Table 1) was corrected for background media-only absorbance where after the 50% cell killing drug concentration (IC_{50} value) was calculated using SigmaPlot® (SPSS Science, Dyrberg Trading, Denmark).

Recombinant enzymes. Generation, recombinant expression and purification of wild type Dm-dNK and of Dm-dNKΔ20 (with a C-terminal deletion) has been described by Munch-Petersen et al. [13].

Enzyme assay. Deoxyribonucleoside kinase activities were determined by initial velocity measurements based on four time samples by the DE-81 filter paper assay using tritiumlabeled nucleoside substrates as described previously [13].

Analysis of kinetic data. Kinetic data were evaluated by nonlinear regression analysis using the Michaelis–Menten equation $v = V_{max} \times [S]/(K_m + [S])$ as described in [14]. The equations were fitted to all available data in a global fit. K_m is the Michaelis constant [15,16]. The kinetic parameters reported here are the average ± SD (standard deviation) obtained from 5 independent measurements.

Crystallization. Crystals of a C-terminally truncated Dm-dNK, lacking the last 20 amino acid residues (Dm-dNKΔ20), complexed with gemcitabine were grown using the vapor diffusion method with hanging drops. The crystallization solution consisted of 0.1 M MES pH 6.5, 0.2 M lithium sulphate and 26% mPEG2000. Two microliter of crystallization solution was mixed with 2 μl enzyme solution containing 15 mg/ml enzyme and 5 mM gemcitabine. The solutions were then left to equilibrate at 14 °C and crystals typically appeared after 2 days. They were formed as cubes, rods or diamonds and after 2–3 weeks they had grown to a size of 75 × 75 × 75 μm for the cubes, 200 × 50 × 50 μm for the rods and 150 × 100 × 50 μm for the diamonds. Crystals were mounted in loops and after a quick wash in a cryo-solution (equal to the crystallization conditions containing 27% mPEG2000) flash frozen in liquid nitrogen.

Data collection. X-ray diffraction data were collected at 100 K on beamline ID14-1 at ESRF in Grenoble, France, using an ADSC Q210 CCD detector. Typically, the rods and cube-formed crystals diffracted to 2.5 Å but one diamond shaped crystal diffracted beyond 2 Å in one direction. Since the crystal diffracted anisotropically the data where cut at 2.2 Å to get a complete dataset. The data were processed and scaled using the programs Mosflm [17] and Scala [18]. The diamond shaped crystal, that data were collected on, belong to the orthorhombic spacegroup $P2_12_12$ and have a solvent content of 51.7%, which corresponds to one dimer in the asymmetric unit. Data collection statistics are shown in Table 2.

Structure determination and refinement. The structure was solved by molecular replacement using the program Phaser [18]. The refined structure of the previously solved Dm-dNK dimer complexed with deoxycytidine (dC) was used as search model. After rigid-body and restrained refinements in Refmac5 [18] an initial electron map was calculated. From this map most of the polypeptide chain could be built using the programs O [19] and COOT [20]. The structure has been refined at 2.2 Å resolution to an R_{factor} of 20.7 and R_{free} of 25.3 with good stereochemistry (Table 2), and the PDB code is 2vpp.

Results and discussion

Sensitivity of transduced human cancer cell lines

In this study, we tested Dm-dNK for its potential as a suicide gene in combination with gemcitabine. Two different cancer cell lines were employed: the glioblastoma cancer cell line U-87-MG and the ovarian cancer cell line AG6000. Transduction of AG6000 cells resulted in a 44-fold increase of sensitivity when compared to the parental cells (Table 1) suggesting that Dm-dNK can sensitize cancer cell lines to gemcitabine. Further on, we attempted to elucidate the molecular background for the observed ability to additionally sensitize the cancer cell towards this drug.

Kinetic parameters

Dm-dNK and Dm-dNK mutants have previously been characterized for their kinetic parameters on the native substrates and various analogs [7]. Hereby we tested the recombinant Dm-dNK for its ability to phosphorylate gemcitabine under *in vitro* conditions and

Table 1
Cytotoxic effect of gemcitabine on two cancer cell lines transduced with Dm-dNK. The cell lines used were the glioblastoma cell line U-87M-G and the ovarian cancer cell line AG6000 and the IC_{50} values for gemcitabine are shown The-fold of decrease in IC_{50} compared to the IC_{50} of the un-transduced parental cells is given in brackets. IC_{50} are average from three independent experiments ± SE.

	IC_{50} gemcitabine (in μM)	
	U-87-MG	AG6000
Parental	>100	32.99 ± 3.79
Dm-dNK	29.89 ± 4.27 (3)	0.76 ± 0.066 (44)

Table 2
Data collection and refinement statistics for Dm-dNKΔ20 complexed with gemcitabine.

Space group	$P2_12_12$
Cell dimensions (Å)	a = 62.3
	b = 68.1
	c = 120.1
Content of the asymmetric unit	1 dimer
Resolution (Å)	50.0–2.2
Completeness (%)	99.1 (99.9)
R_{meas} (%)	8.3 (40.8)
Mean (I/SD)	12.0 (3.8)
Redundancy	3.1 (3.1)
Number of observed reflections	497547
Number of unique reflections	24968
ESRF beam line	ID14Eh1
Wavelength (Å)	0.934
Temperature (K)	100
R_{factor} (%)	20.7
R_{free} (%)	25.3
Rmsd bond lengths (Å)	0.011
Rmsd bond angles (°)	1.377
Mean B-value (Å^2)	28.3

determined kinetic parameters. Dm-dNK could indeed phosphorylate gemcitabine with the K_m value of 20.7 ± 3.1 μM and V_{max} of 13,374 ± 1096 pmol min^{-1} mg^{-1}. This translates to a k_{cat} of 6.50 s^{-1} and k_{cat}/K_m of 314,000 M^{-1} s^{-1}. These kinetic parameters of gemcitabine, compared with the Dm-dNK parameters for other pyrimidine analogs, suggest that Dm-dNK is a relatively good activator of gemcitabine. In the previous work, it has been shown that while deoxycytosine (dC) is a very good substrate for Dm-dNK (K_m value of 2.3 μM and k_{cat}/K_m of 7,200,000 M^{-1} s^{-1}), cytosine analogs are not good substrates [13]. Zalcitabine (ddC) has been reported to have the K_m value of 460 μM and k_{cat}/K_m of 736 M^{-1} s^{-1} and Cytarabine (araC) has a K_m value of 32 μM and k_{cat}/K_m of 59,000 M^{-1} s^{-1} [7]. In addition, in comparison with human dCK, having k_{cat}/K_m of 71,000 M^{-1} s^{-1} for gemcitabine [21], Dm-dNK is about 4-fold more efficient in phosphorylating gemcitabine. What could be the reason that Dm-dNK uses gemcitabine more efficiently?

Structure and structure–function relationship

In order to describe the molecular interactions of Dm-dNK with gemcitabine, we determined the crystal structure of a C-terminal truncated version of wild type Dm-dNK (Dm-dNKΔ20) [13] with gemcitabine bound in the active site (Fig. 1, Table 2). In the difference electron density map there is well-defined density for gemcitabine at the substrate site and a sulphate ion bound in the P-loop in both subunits of the dimer (Fig. 2). The position of gemcitabine in the active site of Dm-dNK is practically identical to that of dC

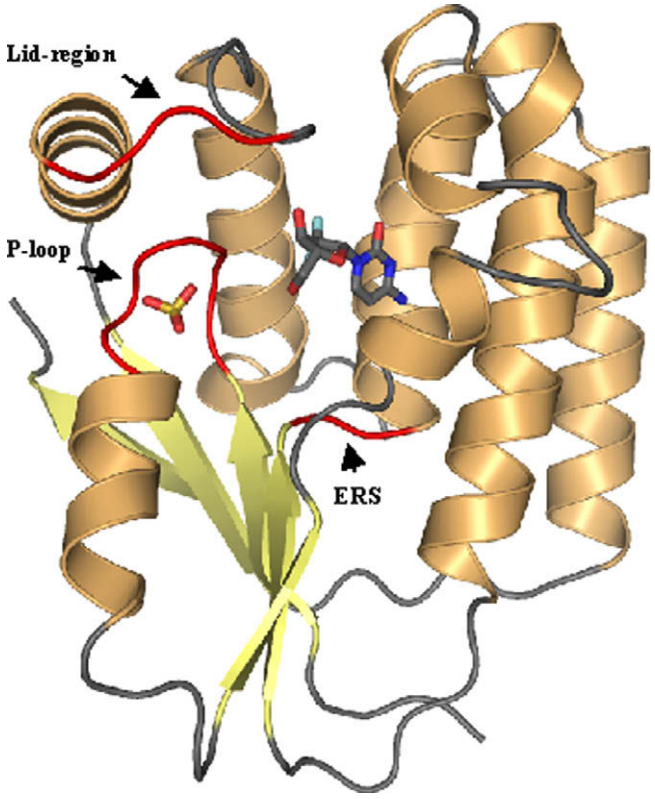

Fig. 1. The subunit structure of Dm-dNKΔ20 with bound gemcitabine in grey and sulphate ion in yellow. Several nucleotide kinases share three common motifs: the conserved P-loop (GXXXXGKS/TT), the arginine rich LID region (RXXXRXRXXE) and the ERS motif. The P-loop is involved in binding and positioning of the α- and β-phosphoryl groups of the phosphate donor. The LID region is a flexible segment containing three conserved arginines which close down on the phosphate donor. The ERS motif is involved in substrate binding and is conserved between dCK, dGK, Dm-dNK, and TK2. The phi/psi angles of the arginine residue in the ERS motif are located in the disallowed area of the Ramachandran plot. The loops containing the P-loop, LID region and ERS motif, are shown in red color. The structure Protein Data Base code is 2vpp.

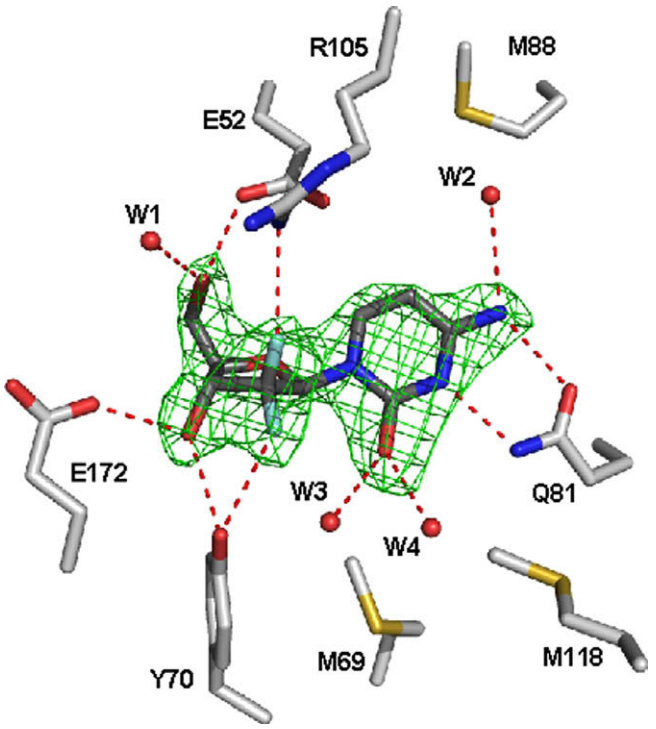

Fig. 2. Positioning of gemcitabine and conserved water molecules in the active site of Dm-dNKΔ20. The difference density for gemcitabine is well defined and contoured at 3σ. Hydrogen bonds are shown as red dotted lines and water molecules as red spheres.

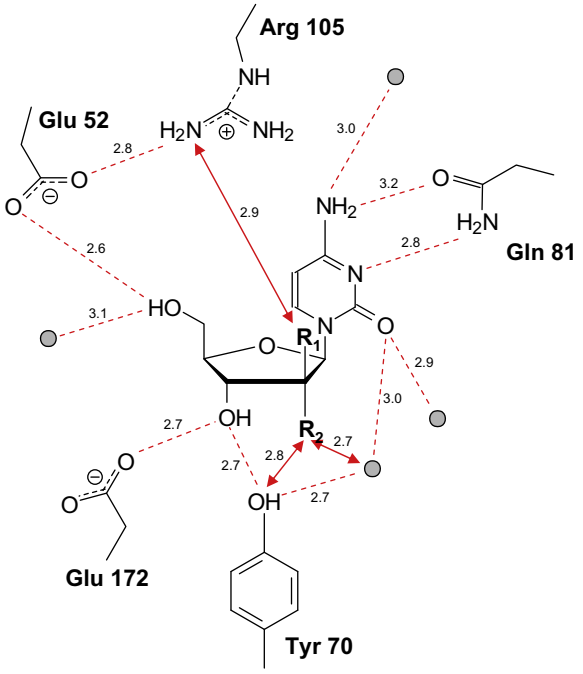

Fig. 3. Schematic representation of the interaction of dC/gemcitabine bound in the Dm-dNKΔ20 active site. For dC R1 and R2 are hydrogen and for gemcitabine R1 and R2 are fluorines. Hydrogen bonds are indicated by red dotted lines and water molecules as grey spheres. Solid red arrows indicate the extra interactions made by the fluorine atoms in gemcitabine.

[22,23]. Gemcitabine has three more favorable interactions due to its fluorine atoms where F1 has a hydrogen bond to NH1 of Arg105 and F2 interacts with both Tyr70 and a water molecule (Figs. 2 and 3). In comparison, ddC has been reported not to be able to make these interactions when bound to the active site of Dm-dNK [22]. This analog misses the 2′- and also 3′-OH group. On the other hand, araC, which is about 5-fold less efficiently converted than gemcitabine, has a 3′-OH group and an OH group in the same position as F1 of gemcitabine, but lacks an equivalent to F2. We therefore postulate that the additional interactions made by the fluoride atoms (Figs. 2 and 3) make gemcitabine a better substrate of Dm-dNK than other cytosine analogs.

Conclusions and outlook

Gemcitabine is an efficient substrate for Dm-dNK. The structural knowledge presented here can be exploited to direct mutagenesis with the aim to create Dm-dNK forms that are more efficient and selective for gemcitabine, and to be subsequently tested for their potential in suicide gene-therapy.

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References

[1] E.S. Arner, S. Eriksson, Mammalian deoxyribonucleoside kinases, Pharmacology & Therapeutics 67 (1995) 155–186.
[2] W. Knecht, G.E. Petersen, B. Munch-Petersen, J. Piskur, Deoxyribonucleoside kinases belonging to the thymidine kinase 2 (TK2)-like group vary significantly in substrate specificity, kinetics and feed-back regulation, Journal of Molecular Biology 315 (2002) 529–540.
[3] W. Knecht, G.E. Petersen, M.P. Sandrini, L. Sondergaard, B. Munch-Petersen, J. Piskur, Mosquito has a single multisubstrate deoxyribonucleoside kinase characterized by unique substrate specificity, Nucleic Acids Research 31 (2003) 1665–1672.
[4] B. Munch-Petersen, J. Piskur, L. Sondergaard, Four deoxynucleoside kinase activities from *Drosophila melanogaster* are contained within a single monomeric enzyme, a new multifunctional deoxynucleoside kinase, Journal of Biological Chemistry 273 (1998) 3926–3931.
[5] W. Knecht, M.P. Sandrini, K. Johansson, H. Eklund, B. Munch-Petersen, J. Piskur, A few amino acid substitutions can convert deoxyribonucleoside kinase specificity from pyrimidines to purines, EMBO Journal 21 (2002) 1873–1880.
[6] W. Knecht, B. Munch-Petersen, J. Piskur, Identification of residues involved in the specificity and regulation of the highly efficient multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster*, Journal of Molecular Biology 301 (2000) 827–837.
[7] W. Knecht, E. Rozpedowska, C. Le Breton, M. Willer, Z. Gojkovic, M.P. Sandrini, T. Joergensen, L. Hasholt, B. Munch-Petersen, J. Piskur, Drosophila deoxyribonucleoside kinase mutants with enhanced ability to phosphorylate purine analogs, Gene Therapy 14 (2007) 1278–1286.
[8] K.W. Culver, Z. Ram, S. Wallbridge, H. Ishii, E.H. Oldfield, R.M. Blaese, *In vivo* gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors, Science 256 (1992) 1550–1552.
[9] F.L. Moolten, Tumor chemosensitivity conferred by inserted *Herpes* thymidine kinase genes: paradigm for a prospective cancer control strategy, Cancer Research 46 (1986) 5276–5281.
[10] X. Zheng, M. Johansson, A. Karlsson, Retroviral transduction of cancer cell lines with the gene encoding *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase, Journal of Biological Chemistry 275 (2000) 39125–39129.
[11] L.P. Jordheim, C.M. Galmarini, C. Dumontet, Gemcitabine resistance due to deoxycytidine kinase deficiency can be reverted by fruitfly deoxynucleoside kinase, DmdNK, in human uterine sarcoma cells, Cancer Chemotherapy & Pharmacology 58 (2006) 547–554.
[12] L. Toschi, G. Finocchiaro, S. Bartolini, V. Gioia, F. Cappuzzo, Role of gemcitabine in cancer therapy, Future Oncology 1 (2005) 7–17.
[13] B. Munch-Petersen, W. Knecht, C. Lenz, L. Sondergaard, J. Piskur, Functional expression of a multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster* and its C-terminal deletion mutants, Journal of Biological Chemistry 275 (2000) 6673–6679.
[14] W. Knecht, U. Bergjohann, S. Gonski, B. Kirschbaum, M. Loffler, Functional expression of a fragment of human dihydroorotate dehydrogenase by means of the baculovirus expression vector system, and kinetic investigation of the purified recombinant enzyme, European Journal of Biochemistry 240 (1996) 292–301.
[15] C. Liebecq, IUBMB Biochemical Nomenclature and Related Documents, Ed., Portland Press Ltd., London, 1992.
[16] A. Cornish-Bowden, Fundamentals of enzyme kinetics, Ed., Portland Press Ltd., London, 1995.
[17] A.G.W. Leslie, in Joint CCP4 + ESF–EAMCB Newsletter on Protein Crystallography, 1992.
[18] Collaborative Computational Project, The CCP4 suite: programs for protein crystallography, Acta Crystallographica Section D-Biological Crystallography 50 (1994) 760–763.
[19] T.A. Jones, J.Y. Zou, S.W. Cowan, M. Kjeldgaard, Improved methods for building protein models in electron density maps and the location of errors in these models, Acta Crystallographica Section A-Foundations of Crystallography 47 (1991) 110–119.
[20] P. Emsley, K. Cowtan, Coot: model-building tools for molecular graphics, Acta Crystallographica Section D-Biological Crystallography 60 (2004) 2126–2132.
[21] T. McSorley, S. Ort, S. Hazra, A. Lavie, M. Konrad, Mimicking phosphorylation of Ser-74 on human deoxycytidine kinase selectively increases catalytic activity for dC and dC analogues, FEBS Letters 582 (2008) 720–724.
[22] N.E. Mikkelsen, B. Munch-Petersen, H. Eklund, Structural studies of nucleoside analog and feedback inhibitor binding to *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase, FEBS Journal 275 (2008) 2151–2160.
[23] K. Johansson, S. Ramaswamy, C. Ljungcrantz, W. Knecht, J. Piskur, B. Munch-Petersen, S. Eriksson, H. Eklund, Structural basis for substrate specificities of cellular deoxyribonucleoside kinases, Nature Structural Biology 8 (2001) 616–620.