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Engineering Corticosteroid-Binding Globulin to Release New Compounds at New Sites

Wee Lee Chan, Aiwu Zhou, Randy J Read. *Department of Haematology, Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Addenbrooke's Hospital, University of Cambridge (United Kingdom).* E-mail: wlc29@cam.ac.uk

Corticosteroid-binding globulin (CBG) is a blood plasma protein that transports the weakly water-soluble hormone, cortisol, throughout the circulation. It is a member of the serine protease inhibitor (SERPIN) structural family; upon cleavage by human neutrophil elastase, it undergoes the canonical S-to-R transition, which results in a change in its binding affinity for cortisol, allowing it to release the hormone at sites of inflammation. We plan to redesign CBG to transport compounds other than its physiological ligand and, by harnessing the S-to-R transition, release them at specific sites in the body.

In preliminary experiments, we succeeded in altering the proteinase specificity of CBG, making it susceptible to cleavage by human α-thrombin. We plan to build on this and prove the principle that by altering the amino acid sequence of the reactive centre loop, CBG can be made to release its ligand in response to specific cleavage by various proteinases. We are especially interested in making it susceptible to proteinases that are tissue-specific so that the engineered protein would be able to deliver its cargo at very specific sites in the body.

We are also looking at re-designing the steroid-binding pocket of CBG so that it is able to bind other compounds with high affinities. Apart from that, it is also necessary to make the change in affinity between the S-state and the R-state much larger should the engineered protein ever be considered for use as a drug-delivery system. In order to study the binding site, and to understand in depth how the change in binding affinity is achieved by the S-to-R transition, it is necessary to obtain crystal structures of CBG in both forms from the same organism as the structures currently available for the native and cleaved forms of CBG are from different organisms [1], [2]. This makes it difficult to tell if the differences in the positions of various residues in the binding pocket are due to conformational changes or a lack of sequence identity.

These structural studies will be complemented with molecular modeling and biochemical experiments to determine how the residues lining the binding pocket contribute to ligand binding and to the mechanism that results in the change in ligand-binding affinity following proteolytic cleavage of the reactive centre loop.

Whilst studying other factors that can affect ligand binding, we also discovered that binding affinity decreases when temperature increases, even within the small range allowed physiologically. This observation was corroborated by a recent study by Cameron *et al* [3]. We think this is another property we can exploit because the local temperature in different parts of the body can vary with tissue type and disease state, and consequently may add another level of specificity for the release of potentially therapeutic compounds from the engineered protein.

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Structure-based design of anti-trypanosomal drugs

Matheus Pinto Pinheiro,[a] Josmar Rodrigues da Rocha,[b] Juliana Cheleski,[b] Helton José Wiggers,[b] Carlos Alberto Montanari,[b] Maria Cristina Nonato,[a] *[a]Laboratório de Cristalografia de Proteínas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo, FCFRP-USP, Brazil. [b]Grupo de Química Medicinal, Instituto de Química de São Carlos - Universidade de São Paulo, IQSC-USP, Brazil.* E-mail: mpp@fcfrp.usp.br

Dihydroorotate dehydrogenase (DHODH) catalyses the conversion of L-dihydroorote (DHO) to orotate, the fourth step and only redox reaction in the *de novo* pyrimidine biosynthetic pathway. The DHODHs can be divided into two major classes on the basis of their amino acid sequences and their cellular location. The enzyme dihydroorotate dehydrogenase (DHODH) has been considered a promising target for the design of trypanocidal agents. Interestingly, trypanosomatids and human DHODH have the different origins, belonging to families 1 and 2, respectively, and display structural differences that can be exploited for the development of selective drugs for the treatment of trypanosomal diseases.

It is our aim to contribute to the development of selective inhibitors for trypanosomal DHODHs. In order to achieve this goal, we have explored the human, *T. cruzi* and *L. major* enzymes, to guide the search for molecules that selectively bind to the trypanosomal enzymes, which is an important rationale behind the design of chemotherapeutic agents.

We report here the discovery of novel inhibitors of Trypanosomatids DHODH identified by virtual screening method and the crystal structure of these ligands in complex with DHODH from *L. major*. The crystal structure of DHODH from *L. major* was solved in the presence of different ligands. Monitoring of the enzymatic reaction in the presence of selected ligands together with structural information obtained from X-ray crystallography analysis have allowed the identification and validation of a novel site of interaction. Our results have provided important structural insights for the rational design of *T. cruzi* and *Leishmania major* DHODH inhibitors.

Keywords: Trypanosomatids, dihydroorotate dehydrogenase, drug design

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Biophysical and biochemical characterization of fumarases from *Leishmania major*

Patrícia Rosa Feliciano,[a] Marcelo Dias Baruffi,[b] Antonio Jose da Costa Filho,[c] Paul A. M. Michels,[d] Maria Cristina Nonato,[a] *[a]Laboratório de Cristalografia de Proteínas - FCFRP - USP, Ribeirão Preto - SP, (Brazil). [b]Departamento de Análises Clínicas, Toxicológicas e Bromatológicas - FCFRP – USP, Ribeirão Preto - SP, (Brazil); [c]Instituto de Física de São Carlos – USP, São Carlos - SP, (Brazil); [d]Research Unit for Tropical Diseases, de Duve Institute, UCL, Brussels, (Belgium).* E-mail: patricia.rf@usp.br

Leishmaniases, classified as neglected tropical diseases, is caused by the parasite *Leishmania* and affect 12 million people in 88 countries around the world.

Fumarate hydratases (FHs) catalyse the stereospecific reversible hydration of fumarate to malate. Eukaryotes express two isoforms of FH, the mitochondrial isoform which performs this reaction as part of the tricarboxylic acid cycle and as such is central to aerobic respiration and the cytosolic isoform which is thought to be involved in the metabolism of fumarate. As a first step aiming the validation of *Leishmania major* FH (LmFH) as target for drug design against leishmaniases, the recombinant enzymes have been used to perform kinetic, biophysical and structural characterization.

Circular dichroism studies have identified differences in secondary

structure content when comparing both isoforms and electron paramagnetic resonance approach has identified the presence of an iron-sulfur cluster. In addition, significant differences in specific activities were found for both isoforms. Polyclonal antibodies have also been raised and subcellular localization studies for both isoforms indicated that one enzyme is localized in mitochondria, whereas the second isoform has double cytosol and glycosome localization. Crystallization and structural determination of LmFH isoforms are in progress.

Our results suggest that LmFH isoforms, which share around 60% of sequence identity, are localized in different cell compartments, and also display differences in protein folding and mechanism of action.

The results, here presented, correspond to the first studies on FHs from trypanosomatides, significantly contributing to the understanding of their functional role in *Leishmania*.

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Keywords: fumarase, leishmania, characterization

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Rational design of inhibitors of APE1 supported by crystallographic techniques

Maria Tintoré,[a] Sandrea M. Francis,[b] Ruben Gil,[c] Antonio Morreale,[c] Angel R. Ortiz,[c] Federico M. Ruiz,[d] Carme Fábrega,[a] [a]*Institute for Research in Biomedicine of Barcelona, Barcelona (Spain).* [b]*Institute of Biomedicine of Valencia (IBV-CSIC) Valencia (Spain)* [c]*Bioinformatics Unit, CBMSO (CSIC-UAM), Madrid, (Spain)* [d]*Chemical and Physical Biology CIB (CSIC) Madrid (Spain).* E-mail: maria.tintore@irbbarcelona.org

Chemotherapy still constitutes the major pharmacologic approach against cancer. However, the biochemical repair systems of the cancer cell machinery responds, trying to mitigate the cellular damage induced by these agents. As a result, the clinical efficacy of chemotherapeutic agents is often limited. Several advances in the molecular biology of cancer have identified key pathways involved in the DNA repair of the damage induced by chemotherapeutic agents. Between all the mechanisms, we can highlight the base excision repair (BER) pathway. Apurinic/apyrimidinic endonuclease (APE-1) is one of the crucial enzymes in this mechanism. Due to its activity, further studies have been focused in the development of inhibitors for APE-1 enzyme. Here, we report the employment of docking and virtual screening techniques based in the crystallographic models to search for compounds that can act as APE-1 inhibitors. The discovered compounds have shown to be active *in vitro* assays, with activities between low and medium micro-molar range. Currently, we are undertaking *in vivo* assays in order to determine their cytotoxicity and their effects in cancer cell survival. At the same time, we are in the process of solving the crystallographic structure of the complexes formed by APE-1 and each one of the active compounds in order to optimize their affinity for the target.

Key words: inhibitor, complex, structure

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Structural studies of *E.coli* nitroreductase enzymes for use in gene therapy of cancer

Martin A. Day,[a,b] Peter F. Searle,[a] Eva I. Hyde,[b] Scott A. White,[b] [a]*School of Cancer Sciences,* [b]*School of Biosciences, University of Birmingham, (UK).* E-mail: mad571@bham.ac.uk

The nitroreductase enzymes of *E.coli* have potential for use in Virus-Directed Enzyme Prodrug Therapy (VDEPT), converting the inactive prodrug CB1954 (5-[aziridin-1-yl]-2,4-dinitrobenzamide) to a potent difunctional alkylating agent [1]. The combination of the minor nitroreductase NfsB and the prodrug CB1954 has been put through Phase I/II clinical trials with promising results. The VDEPT uses an adenovirus vector to insert the gene encoding the nitroreductase into tumour cells, and then the prodrug is separately administered, and upon reaction first with the enzyme and subsequently with cellular thioesters, is transformed into a cell killing agent. This compound is able to transfer into nearby cells and kill those too, causing a 'bystander effect', which is essential for the effectiveness of the treatment. However for treatment to be successful greater efficacy is required.

The major limiting factor is the relatively poor affinity of NfsB for CB1954. Mutants of NfsB have been produced which bind more strongly to the prodrug. It has also been discovered that NfsA, the major *E.coli* nitroreductase, confers 3.5-8 times greater sensitivity to CB1954 than NfsB, in human cells and bacterial cells [2]. The crystal structures of two of the most active NfsB mutants, a double mutant (T41LN71S) and a triple mutant (T41QN71SF124T) are presented here in complex with different inhibitors and compared with wild-type [3]. These structures help us to understand the improvements in binding seen with these mutants. The cooperativity of the T41Q and F124T mutations is shown through a direct H-bonding interaction between the side chains and then to the inhibitor, providing an explanation for why the combination of these mutations has a greater effect than would be expected by the combination of the improvements seen with the single mutants alone.

Also presented are crystal structures of NfsA bound both to inhibitors and to a substrate, the antibiotic nitrofurantoin. The two nitroreductase enzymes are both homodimeric flavoenzymes, with two FMN containing active sites at the dimer interface. Despite having little sequence homology they do share several structural similarities. These structures can be used to guide future rational mutagenesis of the enzymes and design of improved prodrugs, ultimately creating an effective treatment method.

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Crystal Structures of PPARα in Complex with Synthetic and Natural Ligands

Amanda Bernardes,[a] André S. Godoy,[a] Daniela B. B. Trivella,[a] João Renato C. Muniz,[b] Igor Polikarpov,[a] [a]*Department of Physics, University of São Paulo, São Carlos, (Brazil).* [b]*Structural Genomics Consortium, Oxford University, Oxford, (England).* E-mail: abernardes@ursa.ifsc.usp.br

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptors superfamily, a group of transcription factors. Three subtypes are encoded by separate genes: PPARα, PPARβ/δ, and PPARγ, each with different ligand specificity, very distinct tissue distributions, and different biological functions. They are involved in numerous physiological events in human, including glucose and lipid metabolism. PPAR ligands effectively treat dyslipidemia and have significant anti-inflamatory and antiatherosclerotic activity. These effects and their ligand-dependent activity make nuclear receptor obvious targets for drug design in many therapeutic areas [1]. The ligands may be classified in synthetic compounds, such as