MICROBIOLOGY

Overcoming antibiotic resistance

One mechanism of antibiotic resistance in bacteria is through the action of efflux pumps that lower the intracellular concentrations of antibiotics by transporting them to the outside of the cell. Many Gram-positive bacteria use efflux pumps belonging to the major facilitator superfamily, including NorA of *Staphylococcus aureus*, PmrA of *Streptococcus pneumoniae*, and EmeA of *Enterococcus faecalis*. Efflux pumps from this superfamily, sometimes referred to as multiple drug resistance (MDR) pumps, are also found in eukaryotic cells. Inhibition of efflux pumps as a way to improve the clinical efficacy of an antibiotic is a research area of great interest. One such inhibitor, called reserpine, has been identified and shown to inhibit efflux activity in mammalian and bacterial cells, but is limited in its clinical utility due to its neurotoxicity.

In a recent paper by Mullin *et al*., the authors show that two new MDR pump inhibitors, VX-710 and VX-853, which have been shown to be active against mammalian efflux pumps, are potent inhibitors of Gram-positive bacterial MDR pumps as well [1]. Using ethidium bromide (EtBr) as an antibiotic, the authors show that the presence of VX-710 and VX-853 lowers the minimal inhibitory concentration (MIC) of EtBr for *S. aureus, S. pneumoniae*, and *E. faecalis*. The authors also demonstrate that VX-710 and VX-853 directly inhibit EtBr efflux from *S. aureus* to show that the action of the drugs affects the expected resistance mechanism.

To show that these MDR inhibitors are able to potentiate the activity of a broad range of antibiotics, the authors demonstrate that VX-710 and VX-853 lowered the MICs of other antibiotics such as levofloxacin, ciprofloxacin, norfloxacin, gentamicin, novobiocin, tetracycline and tetraphenylphosphonium bromide in certain test strains of *S. aureus*. However,

VX-710 and VX-853 could not lower the MIC for ciprofloxacin and norfloxacin in some clinical isolates of *S. aureus* and *E. faecalis* that display high-level resistance to this class of antibiotics (the fluoroquinolones).

Thus, VX-710 and VX-853 show exciting potential as useful MDR inhibitors, but more characterization of these compounds is necessary before they can be considered for widespread use. Studies such as the one described here highlight the existence of different avenues that can be explored in the fight against antibiotic resistance.

1 Mullin, S. *et al.* (2004) Inhibition of antibiotic efflux in bacteria by the novel multidrug resistance inhibitors Biricodar (VX-710) and Timcodar (VX-853). *Antimicrob. Agents Chemother.* 48, 4171–4176

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MOLECULAR BIOLOGY

53BP1 is recruited to DNA double-strand breaks by binding to histone H3

It is essential to repair DNA double-strand breaks (DSBs) to maintain the integrity of the genome. However, it is still not clear how DSBs are detected. 53BP1 is a DSB sensor and Huyen *et al.* have now shown that it is recruited to DNA breaks by binding to a methylated lysine on histone H3 [2].

The authors solved the structure of the domain of 53BP1 that recruits it to DSBs. It consisted of two tudor folds with a cleft between the two that contained several highly conserved residues. The functional importance of these residues was tested using site-directed mutagenesis. The mutant proteins were fused to GFP and their localization followed *in vivo*. Recruitment to DNA breaks was reduced in all the proteins containing mutations in the cleft, showing that this region targets 53BP1 to DSBs.

The tudor fold has been shown to interact with methylated amino acids, so the authors tested whether the physiological target of 53BP1 is methylated histones. Using GST pulldowns they found that 53BP1 specifically bound to histone H3, but not when H3 was expressed in bacteria, suggesting that posttranslational modification was important. The only modification detected in fragments bound to 53BP1 was methylation at Lys79 and this interaction was confirmed using synthetic peptides. To show that methylation is important *in vivo* the authors reduced the expression of the Lys79 methylase, DOT1L, using siRNA and found that this inhibited 53BP1 recruitment to DSBs.

The authors showed that the methylation level of Lys79 did not change in response to DNA damage, suggesting that it is exposure of already methylated Lys79 rather than increased methylation that recruits 53BP1 to DNA breaks. One possibility is that the DSBs cause a change in the chromatin structure, possibly due to localized relaxation of DNA supercoils, to expose Lys79 of histone H3.

2 Huyen, Y. *et al.* (2004) Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. *Nature* 432, 406–411

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IMMUNOLOGY

DC-SIGN enhances HIV-1 transmission from DCs to T cells through virological synapses

HIV transmission worldwide occurs mainly through sexual contacts. Small amounts of virus cross mucosal surfaces and are captured by dendritic cells (DCs) to reach replication-competent sites in lymphoid tissue. In addition to classical HIV receptors and coreceptors, (CD4, CCR5 and CXCR4), DCs express a type II transmembrane protein with an external C-type lectine domain, DC-SIGN, that captures and transmits HIV to CD4+ T cells. After encountering CD4+ T cells, virions internalized by DCs relocalize to sites of contact with T cells called infectious synapses.

Arrighi *et al*. investigate the role of DC-SIGN on primary DCs in X4 HIV-1 capture and transmission [4]. Using small interfering RNA-expression lentiviral vectors to specifically knockdown DC-SIGN, they show first that DC-SIGN contributes to HIV binding on DCs, but not to HIV capture and internalization, as similar level of viral capture was measured in DC-SIGN+ and DC-SIGN– DCs.

They show next that DC-SIGN increases transfer of HIV infectivity from DCs to target T cells in trans. Indeed, both DC-SIGN+ Raji cells and primary human DCs are very efficient in transferring HIV to target T cells, by contrast to their DC-SIGN- homologues. The

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CANCER BIOLOGY

Keeping in contact

Contact inhibition is the phenomenon of cessation of cellular proliferation in response to physical contact with other cells at confluence. Loss of contact inhibition is a hallmark of neoplastic transformation.

To identify genes able to repress loss of contact inhibition, Kim *et al.* have devised a novel screen [3]. Rat1A cells were engineered to express a Tamoxifen-inducible N-Myc oncogene (*MycNER*). Without Tamoxifen, these cells are contact inhibited; however, induction of N-Myc results in overgrowth of the monolayer. Constitutive expression of a cDNA library in this cell line allowed selection of genes that suppressed loss of contact inhibition. Genes directly inhibiting proliferation conferred a growth disadvantage and were eliminated from the screen. Conversely, cells that lost contact inhibition due to N-Myc activity proliferated at confluence and were killed by cytotoxic drugs. Cells surviving this process were proposed to have suppressed this phenotype.

The screen identified nine suppressors, including ING4. In the presence of Tamoxifen, ectopic expression of ING4 suppressed loss of contact inhibition and conferred cytotoxic drug resistance to the MycNER cells. However, reduction of ING4 resulted in cell death. Additionally, expression of ING4 in T47D cells inhibited growth in soft agar. These results implicate ING4 as a candidate tumour suppressor gene. Although the overall expression level was largely unaffected, mutations of ING4 were present in cell lines and primary tumours. One common mutation potentially affects sub-cellular localization and, possibly, function. Moreover, the ING4 locus was deleted in 10–20% of human breast cancers.

Loss of contact inhibition and growth in soft agar are cardinal features of transformation. The precise mechanism by which ING4 inhibits these activities is unclear. However, the screening procedure described in this report has a potential application for identifying additional tumour suppressor genes.

3 Kim, S. *et al.* (2004) A screen for genes that suppress loss of contact inhibition: identification of ING4 as a candidate tumor suppressor gene in human cancer. *Proc. Natl. Acad. Sci. U. S. A.* 101, 16251–16256

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enhancement of viral transmission occurs downstream from viral capture, because viral capture is equal in DC-SIGN– and DC-SIGN+ DCs in this system. Further microscopy experiments show that DC-SIGN is not required for the formation of DCs-CD4+ T cells clusters but promotes the formation of infectious synapses between DCs and CD4+ T cells.

Overall, the authors show that DC-SIGN promotes the formation of infectious synapses between DCs pulsed with X4-HIV and resting CD4+ T cells, thereby allowing optimal transfer of HIV infection from DCs to T cells. Whether DC-SIGN has a similar function in the transfer of HIV using CCR5 from DCs to T cells remains to be established. Although the precise function of DC-SIGN in this process remains also to be determined, the rapid kinetics (5–10 min) of HIV transfer to infectious synapses, the absence of colocalization of internalized HIV with DC-SIGN and the presence of DC-SIGN in infectious synapses argue in favour of a signalling model in which DC-SIGN could act as a sensor at the surface of DCs. Upon contact with T cells, DC-SIGN could rapidly send reverse signals to the DCs that allow internalized antigen to be presented at the DC surface.

4 Arrighi, J-F. *et al.* (2004) DC-SIGN-mediated infectious synapse formation enhances X4 HIV-1 transmission from dendritic cells to T cells. *J. Exp. Med.* 200, 1279–1288

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Direct antibacterial activity of complement peptide

The complement system is an important part of the defense against invading microorganisms. Complement is activated either directly by foreign substances/surfaces through the alternative or lectin pathway, or via the classical pathway that enhances the activity of specific antibodies generated against previously encountered pathogens. All pathways converge at complement factor 3 (C3), which is proteolytically cleaved in to several active fragments with a number of proinflammatory effects. One of the released fragments, C3a or anaphylatoxin, is one of the most potent ones. C3a activity is efficiently controlled by an inactivating plasma protease that removes one C-terminal arginine, generating C3a-desArg.

Because C3a is structurally related to antimicrobial peptides (AMPs), Nordahl *et al.* [5] investigated whether C3a or C3a-desArg could function as AMPs. Radial diffusion assays revealed that both molecules inhibited growth of the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* as well the Gram-positive *Enterococcus faecalis.* Also, C3a and C3a-desArg bind to heparin, which is a common feature of cationic AMPs. C3a and C3a-desArg disrupts plasma membranes of *P. aeruginosa,* and permeabilize liposomes. Smaller synthetic peptides spanning the four helical regions of C3a also function as AMPs. Incubation of wound fluid with neutrophils, or purified C3a with neutrophil elastase, generates peptides with similar activity as the synthetic peptides, indicating that C3a-derived AMPs are generated during inflammation.

This study describes for the first time direct antimicrobial activity from peptides generated during complement activation and provides a novel link between complement and AMPs, both important for innate immunity. The discovered AMP activity of C3a could partly explain the increased susceptibility to bacterial infections seen in patients with C3-defiency. Furthermore, these findings could aid in the rational design of novel AMPs for clinical use.

5 Nordahl, E.A. *et al.* (2004) Activation of the complement system generates antibacterial peptides. *Proc. Natl. Acad. Sci. U. S. A.* DOI:10.1073/pnas.0406678101 (Epub. ahead of print; http://www.pnas.org)

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TARGETS AND MECHANISMS

Mutations that cause Parkinson's disease identified

According to the World Health Organization, Parkinson's disease (PD) is the second most prevalent neurodegenerative disease, inflicting four million individuals worldwide. Although most occurrence of PD appears to be sporadic with unknown etiology, rare familial forms of parkinsonism have provided significant insight into the molecular mechanisms underlying the pathogenesis of PD. For instance, α-synuclein aggregation and extensive Lewy bodies are pathological features shared both by sporadic and by dominantly inherited forms of PD caused by point mutations or multiplication of the α-synuclein gene. The identification of genes implicated in PD has therefore received much impetus by medical researchers toiling on understanding the disease process.

In reports published in *Neuron*, Paisán-Ruíz *et al.* [6] and Zimprich *et al.* [7] independently identified mutations in *PARK8/LRRK2* as causative for a familial form of parkinsonism. By utilizing

sequence, single nucleotide polymorphism (SNP), haplotype, linkage and northern blot analysis, these groups identified missense mutations R1441G, R1441C, Y1699C, I1122V and I2020T segregating with PD with high penetration. Furthermore, these mutations were absent in over 1000 control individuals, indicating that these are pathogenic mutations and not mere polymorphisms.

The gene product of *PARK8/LRRK2* is dardarin/LRRK2, predicted to consist of 2527 amino acids encompassing 12 leucine-rich repeats, a tyrosine kinase-like domain, a RAS/small GTPase superfamily domain and a WD40 domain. It is of particular interest that the mutations R1441G and R1441C are within the GTPase domain, given that Ras-like small GTPases can act as molecular switches regulating gene expression, vesicle trafficking, nucleocytoplasmic transport, mitogenic signaling as well as microtubule organization. Although speculative at this point, it is possible that at least some of these cellular functions are affected by the mutation of the conserved arginine residue. Furthermore, the mutation I2020T is within the kinase domain. Another attractive hypothesis proposes that LRRK2 is responsible for the phosphorylation of α-synuclein, thereby playing a key role in the deposit of this protein in dying neurons.

By delineating the role of LRRK2 in the pathogenesis of PD, we will be closer to defining the underlying mechanism for this multifactorial disease. Insights into the disease process would enable us to select druggable targets based on rational molecular approaches. The identification of disease-segregating mutations in *PARK8/LRRK2* has indeed opened up new doors for the search of therapeutics that would prevent or ameliorate PD.

6 Paisán-Ruíz, C. *et al.* (2004) Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron* 44, 595–600

7 Zimprich, A. *et al.* (2004) Mutations in LRRK2 cause autosomal-dominant Parkinsonism with pleomorphic pathology. *Neuron* 44, 601–607

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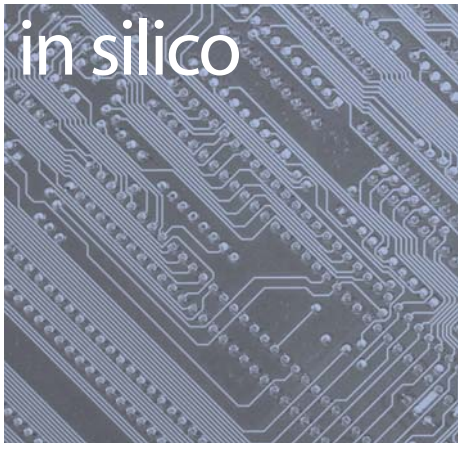

Beware generalizations!

In 2002, researchers at GSK in Pennsylvania published the results of an analysis of rat oral bioavailability (%F) data for over 1100 drug candidates. One of the conclusions from their work was that 80% of compounds meeting two simple criteria showed a %F value of greater than or equal to 20%. The two criteria were that a compound should possess 10 or fewer rotatable bonds and have a polar surface area of 140 Å^2 or below. In the wake of Lipinski's 'rule-of-five', such simple rules-of-thumb have been eagerly seized on because they appear to offer a rapid means of prioritizing compounds at an early stage of drug discovery.

However, in a recent publication [1], a group of scientists from the former Pharmacia site in Kalamazoo has performed a similar analysis on rat %F data for 434 Pharmacia compounds and arrived at some interesting conclusions. First, perhaps obviously to computational scientists, the precise values that are obtained for the number of rotatable bonds in a molecule and its polar surface area depend upon the software that is used to compute these quantities. So, unless it is certain that the software used is the same as in the work used to derive the 'rule', then there is always the danger that a compound will be misclassified.

Second, even when the Pharmacia scientists tried to mimic the GSK calculation procedure as closely as possible, they observed that the percentage of their compounds with %F >20 that satisfied the two criteria was only 70%, compared with the 80% figure reported by the GSK group. Furthermore, the Pharmacia group noticed the results varied depending on the therapeutic target against which the compounds were directed.

The overall conclusions from the work were twofold: (1) it is likely to be extremely difficult to obtain a simple and general rule to predict a complex physiological endpoint such as oral bioavailability; (2) that great caution should be exercised when prospectively applying conclusions obtained from one set of data using one particular computational protocol to other data sets using different protocols.

1 Lu, J.J. *et al.* (2004) Influence of molecular flexibility and polar surface area metrics on oral bioavailability in the rat. *J. Med. Chem.* 47, 6104–6107

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Unravelling the ORFan mystery

With whole-genome sequencing projects progressing fast, studies are well underway to elucidate evolutionary relationships among different organisms on a molecular basis. However, ~20–30% of newly identified open reading frames (ORFs) from a sequenced genome lack any detectable sequence similarity to known ORFs in other species. The number of these orphan ORFs – so called ORFans – can rise up to 60% as in the case of the malaria parasite *Plasmodium falciparum*, making functional and structural assignment difficult using common bioinformatics approaches. ORFans become increasingly abundant in sequence databases with >30,000 representatives to date. Much has been speculated about their origins and roles proposed ranging from 'sequencing errors' to 'non-expressed pseudogenes' to 'unique proteins with new function/3D structure' as few ORFans have been studied experimentally.

Siew and Fischer are challenging this ORFan mystery, describing the impact of structural biology on ORFan research by analyzing recent PDB entries for sequence homology to known proteins [2]. They identified 11% of these as ORFans from various Kingdoms (Archea, Bacteria, Eukarya) and viruses. This suggests that: (1) most ORFans are likely to be expressed into functional proteins; and (2) ORFans are already commonly studied experimentally and more frequently than previously expected. In fact, ~75% of these identified ORFans are already functionally characterized, many assuming roles in transcription and/or translation.

In terms of structure the researchers found that most ORFans have a known fold. The team questioned if fold-recognition methods would have been able to predict those structures with a common fold and found that 30% were correctly predicted when compared with the experimental structure. These findings highlight once more that structure is more conserved than sequence in evolution and the key to function prediction and unfolding evolutionary origins.

The snapshot survey on structural ORFan research undertaken by these authors demonstrates the impact of structural biology on assigning functions and unravelling evolutionary relationships and origins for putative genes discovered through genome-sequencing projects without significant sequence relationship to known genes. Large-scale structural studies are needed to characterize the remaining thousands of ORFans and will show if ORFans are promising source of novel folds and attractive targets for further studies.

2 Siew, N. and Fischer, D. (2004) Structural biology sheds light on the puzzle of genomic ORFans. *J. Mol. Biol.* 342, 369–373

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