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STEROIDS

Building biosynthetic routes in yeast

In 1952, it involved around 40 steps of synthesis. Now, hydrocortisone production can be completed without any chemical synthesis at all by engineering the yeast *Saccharomyces cerevisiae* to biosynthesize the complex product from scratch, according to new research in the February issue of *Nature Biotechnology*.

Hydrocortisone is the major adrenal mammalian glucocorticoid and is an important intermediate in steroid drug synthesis that is used in large quantities in industry. Although the original chemical synthesis of the molecule was very cumbersome, hydrocortisone manufacture now requires just nine steps, including some bioconversions. In mammals, biosynthesis takes place in the adrenal cortex in two different compartments, the ER and the mitochondria. The pathway starts in the mitochondria with the cleavage of the cholesterol side chain to make pregnenolone, which then moves to the ER where it is oxidized and hydroxylated by three more enzymes to make 11-deoxycortisol, before moving back to the mitochondria for the final conversion to hydrocortisone.

Yeast neither makes cholesterol, nor takes up exogenous sterols from the medium during aerobic respiration. In order to produce molecules resembling cholesterol from a simple carbon source, such as glucose or ethanol, biosynthesis of its major sterol, ergosterol, was rerouted using the plant enzyme Δ7 reductase. The resulting products, campesterol and brassicasterol, were converted to pregnenolone by heterologous mitochondrial enzymes. Additional engineering mimicked the adrenal biosynthesis of pregnenolone to hydrocortisone (through intermediates progesterone, 17-hydroxyprogesterone and 11-deoxycortisol) and involved mainly membrane-bound enzymes: overall, three members of the P450 superfamily of monooxygenases, 3β-hydroxy steroid dehydrogenase/isomerase and three electron carriers were expressed. During the conversion, two major unwanted side reactions were identified: the esterification of pregnenolone and the ketoreduction of 17-hydroxyprogesterone. The endogenous yeast genes responsible for these changes were inactivated, bringing the total number of engineered genes in a single micro-organism to 13. The major sterol product of the engineered yeast was hydrocortisone (up to 70% of total steroids), with some by-product of 11-deoxycortisol and corticosterone.

This work represents the most complex engineered pathway described to date and the first example involving several coupled membrane enzymes in a eukaryotic cell. Such engineered microbes could be developed for low-cost industrial processes to substitute chemical approaches in the synthesis of corticoid drugs. In addition, they represent a powerful tool for deciphering steroid synthesis regulation and balance.

Melanie Brazil

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WEB SITES
Encyclopedia of Life Sciences:
http://www.els.net
History of cortisone and related compounds

INFLAMMATORY DISEASES

Setting a trap for cytokines

Considerable advances in treating autoimmune diseases have been made with agents that block the action of cytokines. New work published in the January issue of *Nature Medicine* describes the design and production of a novel class of cytokine antagonists, termed cytokine traps, which overcome some of the problems with existing antagonists.

The best results to date with cytokine antagonists have been achieved using soluble decoy receptors that bind and block the cytokine of interest. The tumour necrosis factor-α (TNF-α) blocker etanercept (Enbrel), which is made up of the extracellular ligand-binding portion of the TNF-α receptor fused to the constant region (Fc) of a human immunoglobulin (Ig) chain, has been approved for the treatment of rheumatoid arthritis (RA), and has good clinical activity. However, for most cytokines, two distinct receptor components cooperate to bind the cytokine very tightly, and so a single-component receptor antagonist does not work very well. Perhaps because of these limitations, single-component soluble receptor antagonists for IL-1 and IL-4 have not been successful.

In an effort to overcome problems of low binding affinity, and short half-life (which necessitates frequent injections), Stahl and colleagues developed soluble cytokine receptors, termed traps, that incorporate both of the receptor components normally required to achieve high-affinity binding. The traps were constructed by engineering linear fusions of both receptor extracellular domains followed by the human IgG1 Fc domain. The Fc portion directed the formation of disulphide-linked dimers. The affinity of cytokines for the traps is significantly greater than for their individual cellular receptor components. IL-1, IL-4 and IL-6 traps were able to

ANTIFERTILITY DRUGS

Fast-track to a male pill?

None of the available options for male contraception, such as condoms, vasectomy and withdrawal, offer the ideal combination of convenience, effectiveness and reversibility. And although considerable efforts have been directed at developing a reversible oral contraceptive drug for men, those drugs closest to the market, which target hormones such as testosterone that are involved in the production of sperm, can cause side effects including weight gain. Now, findings by van der Spoel *et al.*, reported in the *Proceedings of the National Academy of Sciences*, indicate that treatment with a drug recently approved for Gaucher's disease, a rare genetic disorder, might also be a convenient non-hormonal approach to reversible male contraception.

Male mice orally dosed with the drug — the alkylated iminosugar *N*-butyldeoxynojirimycin (*N*B-DNJ) — became sterile after three weeks of treatment, whereas female mice were unaffected. Drug treatment did not influence levels of testosterone, or levels of the reproductive hormones luteinizing hormone and follicle-stimulating hormone, indicating that *N*B-DNJ acts in a non-hormonal manner. And once taken off the drug, male mice regained their fertility after three weeks, and their resultant offspring developed normally.

Sperm from drug-treated mice show several morphological abnormalities, and have severely impaired motility. But how might the drug work at the molecular level? *N*B-DNJ is a well-characterized inhibitor of ceramide-specific glucosyltransferase, a key enzyme involved in the production of glucosphingolipids (GSLs) present on the membrane of all mammalian cells. It is this activity that gives *N*B-DNJ efficacy in Gaucher's disease, which is caused by a build-up of GSLs owing to defects in an enzyme involved in GSL degradation — by reducing GSL synthesis, *N*B-DNJ acts to restore the proper balance. GSLs are also important for sperm production, as mice deficient in enzymes involved in GSL biosynthesis are sterile, leading the authors to suggest that the impact of *N*B-DNJ on male fertility might be caused by altering GSL metabolism during sperm development.

Given the highly conserved nature of sperm production in mammals, it seems plausible that *N*B-DNJ could have analogous effects in man. And because *N*B-DNJ has already been extensively studied in the clinic, it could be rapidly evaluated for its suitability as a male contraceptive. In trials so far, the major side effect of *N*B-DNJ has been dose-dependent diarrhea, but if the very low doses needed to cause infertility in mice were reflected in humans, such side effects might be negligible.

Peter Kirkpatrick

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bind the specific cytokine *in vitro* with high affinity and potently block cytokine action. *In vivo*, mouse versions of the IL-1 and IL-4 traps were able to block the development of arthritic joints in a mouse arthritis model and to prevent the high numbers of eosinophils that accumulate in mouse models of asthma, respectively. The human IL-4 trap was also able to block IL-4 action in primates.

Phase II human clinical trials of the IL-1 trap to assess safety and efficacy in RA patients are underway. Pharmacokinetic data suggest that dosing is compatible with a once-weekly shot, which would be a great improvement over existing treatments, which are injected daily.

Melanie Brazil

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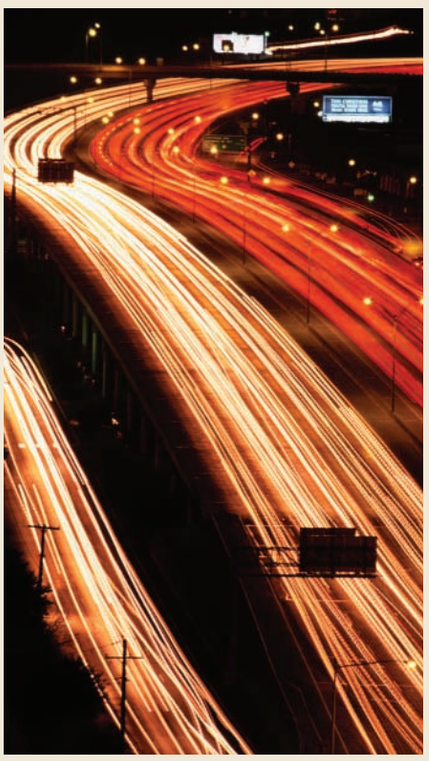

ANTIHYPERTENSIVE DRUGS

ALLHATs off to the golden oldie

"Quite simply, the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) is one of the most important trials of antihypertensive therapy", states Lawrence Appel in the *Journal of the American Medical Association*, in response to the eagerly awaited results from the biggest head-to-head comparison to find the most effective class of hypertension drug. And the results from the trial are striking — the oldest form of treatment seems to be more effective than the newer kids on the block.

Diuretics have been used to treat high blood pressure since the late 1950s. But in the past couple of decades, several classes of treatments with different mechanisms of action have emerged, such as calcium channel blockers (CCBs) and angiotensin-converting enzyme (ACE) inhibitors. These newer treatments have theoretical advantages over diuretics, but with little practical proof of their benefits clinicians have debated which classes of treatment are more likely to work and should therefore be prescribed first to patients.

So, in 1994 a randomized, double-blind trial called ALLHAT, supported by the US National Heart, Lung and Blood Institute, was launched to compare the outcomes of a CCB (amlodipine) and an ACE inhibitor (lisinopril) with a diuretic (chlorthalidone) in over 33,000 hypertensive patients. (Another class of antihypertensive, an α-blocker (doxazosin) was also compared, but was withdrawn during the trial because "it was found to be inferior to the diuretic".) After following the patients for around 5 years, the researchers found that the diuretic was just as effective in preventing the primary end point (the incidence of fatal coronary heart disease (CHD) or nonfatal heart attack) as the other drugs. But in the prevention of secondary outcomes (all-cause mortality, stroke, combined CHD or combined cardiovascular disease), better results and a lower cost gave the diuretic the edge over its counterparts.

The findings from the ALLHAT study will have a great impact on medical care and research. As the study looked at a broad range of people with hypertension, its findings imply that diuretics will be a more appropriate choice over ACE inhibitors and CCBs in almost all cases. And as diuretics cost around 6–20 times less per pill than the other classes, prescribing them first would dramatically reduce healthcare costs (the annual US antihypertensive drug cost is ~$15 billion).

Also, the finding that newer drugs do not necessarily mean better drugs will re-ignite arguments surrounding the approval process.

Approval of a drug only requires proof of efficacy compared with placebo, not whether it works better than existing therapies. In a year that saw another US-government-funded trial disprove the theory that hormone-replacement therapy protects women against heart disease, the findings from the ALLHAT study raise the question of whether one role of governments should be to fund more studies that look into the important medical and economic issues that industry-based trials do not typically address.

Simon Frantz

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ENDOCANNABINOID SYSTEM

FAAH better anxiolytics?

The cannabinoid receptor (CB1), the G protein-coupled receptor activated by the psychotropic components of marijuana, is an attractive target for the development of anxiety- and pain-reducing therapies. However, exogenously applied cannabinoid agonists have been found to give rather variable effects, and a more specific route to regulating CB1 receptor activity might be to find a way of elevating the levels of the endogenous amidated lipids that activate CB1 receptors. Hence there is interest in targeting the principal degradative enzyme for this group of endogenous CB1 receptor agonists, the membrane-bound fatty acid amide hydrolase (FAAH), and to this end recently published papers now report the high-resolution crystal structure for FAAH, and a new class of potent and selective FAAH inhibitors.

Transgenic mice lacking FAAH have previously been shown to have increased levels of anandamide, the main amidated lipid candidate for the role of the 'endogenous cannabinoid', and to show enhanced CB1 receptor-dependent analgesia (see further reading). Now, in the November 29th issue of *Science*, Bracey *et al.* present the 2.8 Å crystal structure of a slightly truncated version of FAAH, complexed with methoxy arachidonyl fluorophosphonate, an active-site-directed inhibitor. The crystallized FAAH variant, despite having the first 29 amino-terminal amino acids deleted, was reported to retain the wild-type enzyme's association with the cell membrane and ability to degrade fatty acid amides. The crystal structure reveals a dimeric enzyme, closely related to malonamidase (MAE2), the other enzyme from the amidase signature family for which a structure has been determined and which hydrolyses malonamide in the nitrogen-fixing bacterium *Bradyrhizobium japonicum*. Interestingly, the close association found in FAAH between the active site and the cell membrane indicates that membrane-permeable lipids, such as anandamide, might not need to be transported through aqueous cellular compartments in order to be degraded, and therefore that the level of endocannabinoid activity might be controlled by the relative expression levels and placement of FAAH and CB1 receptors *in vivo*.

Knowing the structure of FAAH may help with the future design of inhibitors, but meanwhile a paper from Daniele Piomelli's group, published online in December's *Nature Medicine*, reports the discovery of the first selective inhibitors of FAAH. The two most potent compounds in this class of carbamate inhibitors, URB597 and URB532, were able to elicit anxiolytic-like responses in rats without evoking many of the side effects that normally accompany cannabinoid action, and also produced mild anti-nociception in a model of acute pain. FAAH inhibition was shown to result in increased levels not just of anandamide and other amidated lipids that activate CB1 receptors, but also of analogues that act independently of CB1 receptors. So although apparently a new route to the treatment of anxiety by enhancing cannabinoid receptor activation, targeting FAAH also seems likely to affect signalling pathways other than those mediated by CB1 receptors.

Adam Smith

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DIABETES

How DPP-IV takes a bite

Dipeptidyl peptidase IV (DPP-IV, also known as CD26), a multifunctional transmembrane serine protease, has been attracting considerable interest in recent years as a potential target for type 2 diabetes, owing to its role in regulating blood sugar, and initial trials of DPP-IV inhibitors in humans have been promising. The structure of DPP-IV in complex with an inhibitor, reported in the January issue of *Nature Structural Biology*, indicates how substrate specificity is achieved, and should be valuable in understanding the structure–activity relationships of known and future inhibitors, and in achieving high specificity for DPP-IV over other members of the same protein family.

DPP-IV modulates the activity of several neuropeptides, chemokines and peptide hormones by specifically cleaving Xaa-Pro or Xaa-Ala from their amino termini. Cleavage of glucagon-like peptide and glucose-dependent insulinotropic peptide — which together are largely responsible for stimulating insulin secretion by β-cells in response to food — terminates their action. By delaying this normally rapid degradation, DPP-IV inhibitors combat the defects in the effects or production of insulin present in type 2 diabetes. And because the ability of DPP-IV inhibitors to promote insulin release is strongly glucose dependent, the risk of hypoglycaemia, a serious side effect of current therapies, is reduced.

Biochemical evidence indicates that DPP-IV functions as a dimer, and indeed, in the crystal structure of the extracellular part of DPP-IV in complex with the inhibitor valine-pyrrolidide determined by Rasmussen *et al.*, DPP-IV is a dimer. Each monomeric subunit has an α/β hydrolase domain and an eight-bladed β-propeller domain, and both domains participate in inhibitor binding in the active site, as well as in dimerization. Intriguingly, it seems that part of the β-propeller domain involved in dimerization could act as a 'lid' to the active site if the dimer dissociates, which would provide a structural explanation for the biochemical observations that DPP-IV acts as a dimer, and which could represent a novel opportunity for structure-based drug design to block dimer formation.

Peter Kirkpatrick

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ARTHRITIS

Oxygen burst may be crucial

A naturally occurring polymorphism in the protein neutrophil cytosolic factor (Ncf1) regulates the severity of arthritis through a previously unknown mechanism involving arthritogenic T cells, explains an article published in the January issue of *Nature Genetics*. Holmdahl and colleagues used positional cloning to identify the *Ncf1* gene in a particular locus that is associated with arthritis severity. Ncf1, also known as phagocyte oxidase, is a component of the nicotinamide adenine dinucleotide phosphate oxidase (NADPH) complex, which produces reactive oxygen species (ROS). The authors went on to show that pharmacological treatment with drugs that activated the NADPH oxidase complex, thereby producing higher levels of ROS, were able to ameliorate arthritis in rats. Arthritis in the rat model is very similar to rheumatoid arthritis (RA) in humans, so this work suggests new therapeutic pathways to target.

RA is a chronic inflammatory disease that affects peripheral joints; synovial inflammation in these joints leads to cartilage destruction, bone erosion and ultimately joint deformity and loss of joint function. Inheritance of RA is polygenic and influenced by environmental factors. The authors have found, using the rat arthritis model, that different gene regions control different phases of the disease, such as the onset, and severity of the acute and chronic phases.

Ncf1 is expressed in all phagocytic cells, and following phosphorylation it forms part of the NADPH oxidase complex in the cell membrane. This complex has a central role in host defence against bacterial infections through the production of ROS. Holmdahl and colleagues showed that the Ncf1 disease-related polymorphism led to differences in enzyme activity, rather than to quantitative differences in expression, and to a lower oxygen burst, which resulted in more severe arthritis.

High levels of ROS in the joints are believed to be involved in inflammation-mediated joint destruction; however, the possibility that these high ROS levels might actually reduce arthritis severity through earlier events is not usually considered. The authors showed that Ncf1 is involved in the early phase of arthritis due to the generation of disease-causing autoimmune arthritogenic T helper cells. Furthermore, T cells originating from a rat with the disease-related polymorphism could transfer severe arthritis to rats without the Ncf1 polymorphism.

The involvement of Ncf1 in the generation of arthritogenic T cells explains the paradox that a decreased, rather than an increased, oxygen burst is associated with arthritis. Activation of the NADPH oxidase complex is characteristic of activated macrophages and other immune cells that trigger T cells into action. The authors speculate that the production of ROS during T-cell interaction with another immune cell induces apoptosis, which limits the expansion of T cells responding to self-components; a reduction in the ROS allows arthritogenic T cells to escape death.

Melanie Brazil

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WEB SITES

Holmdahl's laboratory: http://net.inflam.lu.se/

Encyclopedia of Life Sciences: http://www.els.net Rheumatoid arthritis

IN BRIEF

LEAD DISCOVERY

Virtual screening to enrich hit lists from high-throughput screening: a case study on small-molecule inhibitors of angiogenin.

Jenkins, J. L., Kao, R. Y & Shapiro, R. *Proteins* **50**, 81–93 (2003)

Hit lists from high-throughput screening (HTS) often contain a large proportion of false positives, which means that follow-up assays are needed to find the truly active compounds. Consequently, there has been growing interest in integrating virtual screening (VS) with HTS with the aim of improving the quality of the hit-lists. Application of such a VS/HTS approach to the discovery of leads against the enzyme angiogenin leads to a sixfold enrichment in the hit rate compared with HTS alone.

GENOMICS

The protein kinase complement of the human genome.

Manning, R. *et al*. *Science* **298**, 1912–1934 (2002)

Using public and proprietary genomic, complementary DNA and expressed sequence tag sequences, the authors identify 518 putative protein kinases in the human genome, providing a starting point for comprehensive analysis of protein phosphorylation in normal and disease states.

HORMONE RECEPTORS

Rapid nontranscriptional activation of endothelial nitric oxide synthase mediates increased cerebral blood flow and stroke protection by corticosteroids.

Limbourg, F. P. *et al. J. Clin. Invest.* **110**, 1729–1738 (2002)

Classically, steroids hormones act by modulating gene expression, with effects occuring over hours to days. However, growing evidence indicates that important effects of steroids are mediated through rapid nontranscriptional mechanisms. Limbourg *et al.* show that the neuroprotective effects of corticosteroids are mediated through rapid non-nuclear activity of the glucocorticoid receptor (GR), suggesting that drugs that selectively activate the nontranscriptional actions of GR might be beneficial in stroke.

GENE THERAPY

Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5.

Qin, X.-F. *et al*. *Proc. Natl Acad. Sci. USA* **100**, 183–188 (2003)

The use of small interfering RNA (siRNA) to reduce expression of specific genes has great therapeutic potential, but a key challenge is delivering siRNA into the target cells. By using a lentiviral vector derived from HIV-1, Qin *et al.* introduced siRNA against the chemokine receptor CCR5 — a necessary co-receptor for infection by HIV-1 — into T cells, which led to up to 10-fold reduction in CCR5 expression, and a 3–7 fold reduction in the number of T cells infected after challenge with HIV-1.

PATENT WATCH

No patent for Harvard oncomouse in Canada

After 17 years in the Canadian court system, the 'Harvard oncomouse' has reached a dead end. The Canadian Supreme Court ruled by a five-to-four decision that the transgenic mouse, genetically engineered with a predisposition to develop cancer, is not an invention and cannot be patented in Canada. Canada is now the only Western nation to deny a patent to the Harvard Mouse. The Canadian Patent Office originally approved Harvard's claim for protection of the process by which the oncomouse was engineered, but denied protection for the mouse itself. This meant that although no one could use Harvard's techniques to produce a new oncomouse without infringing their patent, nothing prevented a third party from obtaining oncomouse offspring by mating an existing oncomouse pair. Harvard's appeal of this decision culminated in the hearing before the Supreme Court. In coming to their decision, the Court focused on two phrases appearing in the Patent Act's definition of invention, namely "manufacture" and "composition of matter". They concluded that "manufacture" should be limited in its interpretation to denote a non-living mechanistic product or process, and thus could not include the oncomouse. In considering the phrase "composition of matter", the Court noted that none of the other words used in the definition of "invention" referred to a higher life form.

WEB SITES

The Supreme Court of Canada: http://www.lexum.umontreal.ca/csc-scc/en/
Harvard College v. Canada
Canadian Patent Act: http://laws.justice.gc.ca/en/P-4/index.html

Markman rules

Following a Markman hearing, the US District Court of Delaware ruled against PolyMASC Pharmaceuticals plc in its patent infringement case against the ALZA Corporation. PolyMASC claims that its patent covering pegylated liposomes for drug delivery is being infringed by ALZA in its manufacture of pegylated-liposome products encapsulating the cancer drug doxorubin. The Markman hearing is a special proceeding required under US patent law in which both sides present to the court their arguments for how they believe certain claims at issue in the lawsuit should be interpreted. The court decides how the patent claim should be interpreted, and this interpretation is used to instruct a jury, should the issue of infringement be reached at trial. Often, the way a court rules on claim construction has a substantial impact on other issues in the case and after the court gives an opinion of the scope of the patent's claims, the outcome of the charge of infringement is inevitable. PolyMASC intends to seek immediate resolution of the current case in the Federal Circuit Court of Appeals, but if the company does not succeed in convincing the appeal court to reverse or modify the Markman ruling, the company's infringement action may not to proceed to trial.

WEB SITE

US Patent and Trademark Office: http://www.uspto.gov/
PolyMASC Pharmaceuticals plc. Liposomes with covalently bound PEG moieties on the external surface which demonstrate improved serum half-life following intravenous administration are provided. US Patent 6,132,763 (2000).

ION CHANNELS

Fluid control

What is the link between the inherited disorder cystic fibrosis and secretory diarrhoea, the biggest killer of children under 5 years of age in developing countries? The answer is the cystic fibrosis transmembrane conductance regulator (CFTR) — a cyclic-AMP-activated chloride channel that is responsible for fluid secretion in the intestines and airways. Mutations in the *CFTR* gene that inactivate the function of the protein lead to cystic fibrosis, and the cholera toxin that causes secretory diarrhoea induces intestinal fluid secretion by affecting CFTR-mediated Cl^- transport. But, so far, the development of treatments that target CFTR has been hampered by the lack of appropriate small-molecule inhibitors to help researchers investigate the relevant pathophysiological mechanisms and potential therapies.

Now, in the *Journal of Clinical Investigation*, Verkman and colleagues report the identification of a class of high-affinity CFTR inhibitors from a screen of 50,000 compounds. They found that six compounds, all thiazolidinones, were potent inhibitors of CFTR-mediated Cl^- transport, and worked in the submicromolar range. The most potent of these inhibitors blocked Cl^- tranport in CFTR, but did not inhibit other Cl^- channels or transporters. This inhibitor was non-toxic in cell-culture and mouse models, and a single dose in mice reduced cholera-toxin-induced fluid secretion by 90% for over 6 hours.

CFTR inhibitors could help advance the development of treatments for secretory diarrhoea and cystic fibrosis in different ways. For secretory diarrhoea, thiazolidinones could provide an alternative line of attack to oral rehydration therapy, which revolutionized the treatment of secretory diarrhoea by single-handedly reducing the mortality of children by more than half. For cystic fibrosis, identifying therapies has been difficult owing to a lack of adequate human-tissue and animal models with impaired CFTR function. But thiazolidinones could at last provide researchers with the much-needed tools to investigate the underlying pathophysiological pathways of this fatal genetic disease.

Simon Frantz

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