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MEETING REPORT

APPROACHES TO ASSESSING DRUG SAFETY IN THE DISCOVERY PHASE

HIGHLIGHTS OF THE SOCIETY FOR MEDICINES RESEARCH SYMPOSIUM HELD ON SEPTEMBER 24TH, 2009, AT THE NATIONAL HEART & LUNG INSTITUTE, KENSINGTON, LONDON, U.K.

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SUMMARY

The Society for Medicines Research symposium, sponsored by Apredica, Gwathmey Preclinical Services, Gentronix and Cyprotex, was held at the National Heart and Lung Institute, Kensington, London, U.K. The meeting, organized by Jack Allen, Phil Jeffrey and Andrew Ratcliffe, focused on approaches to assessing drug safety in the discovery phase. Topics included molecular aspects of adverse drug reactions from molecule to man, the relationship of physicochemical properties to toxicity, reactive metabolites, inhibition of the cardiac sodium channel, drug-induced mitochondrial dysfunction, high-throughput screening for genotoxicity and carcinogenicity, and the use of zebrafish as a model for hepatotoxicity and developmental toxicity.

Attrition in drug development is still cripplingly high, with toxicity the leading cause at all stages in the drug development pipeline. It has been estimated that a 10% improvement in predicting failure before the initiation of expensive and time-consuming clinical trials could save upwards of $100 million in the costs associated with drug development. Furthermore, since approximately 70% of all toxicity-related failures that occur preclinically are comprised of toxicological outcomes for which the preclinical models are predictive of human toxicity, then the benefits of identifying and predicting safety liabilities earlier in the drug discovery and development process could be of enormous benefit and value.

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Professor Kevin Park (MRC Centre for Drug Safety Science, Department of Pharmacology, University of Liverpool, U.K.) delivered the opening lecture on molecular aspects of adverse drug reactions from molecule to man. Professor Park suggested understanding adverse drug reactions at the molecular level, and linking them to pharmacogenomics, offered a way of tailoring the medicine to the individual patient to minimize unwanted adverse toxicity. To illustrate the approach, warfarin therapy was given as an example. Warfarin produces anticoagulation by reducing the binding of coagulation factors to the vascular endothelium. At the mechanistic level, this is driven through inhibition of vitamin K epoxide reductase complex (VKORC1), an enzyme that is a key operator in the post-translational γ-carboxylation of glutamic acid residues on the coagulation factors required for endothelial binding. Warfarin is administered as a racemate, with the more potent (*S*)-isomer undergoing metabolism principally by cytochrome P450 CYP2C9. However, there are certain groups of patients which represent challenges to the dosing and management of warfarin therapy (1-3). Patients with a common functionally defective CYP2C9, resulting in an inability to efficiently eliminate (*S*)-warfarin from the systemic circulation through metabolism, run a higher risk of life-threatening bleeding. To compensate, a significantly lower maintenance dose regimen is required. There are also patients with VKORC1 polymorphisms, which can result in either warfarin sensitivity, as in the case of CYP2C9 polymorphism, or warfarin resistance, in which patients are stratified to higher dose requirements. As a consequence of understanding in detail the mechanism of a drug's action, and associated genetic and environmental factors that avoid adverse drug reactions, new regulatory

labeling can be approved and subsequently used by physicians to deliver improved healthcare. In the case of warfarin, new labeling was approved in 2007, in which genetic variations of CYP2C9 and VKORC1, along with other factors, should be considered when prescribing the drug, although such tests should not delay initiating therapy.

In another example, the importance of mechanistically understanding at the human level the conversion of prodrugs to their active form, and placing in context the risk of adverse drug reactions, was demonstrated in comparing and contrasting the clinically approved $P2Y_{12}$ receptor antagonists clopidogrel (**1**) and prasugrel (**2**) (4, 5). Although both drugs require biotransformation to generate active thiols that subsequently bind irreversibly to cystine residues in the $P2Y_{12}$ ADP receptor on platelets, the pathways to such species differ (Fig. 1).

Through clever design, the ester group in prasugrel is hydrolyzed using intestinal hydroxy esterases to the thiolactone (**3**), which is subsequently metabolized primarily by CYP3A4 to its active thiol (**4**). In contrast, clopidogrel is transformed to its active thiol (**5**) via an intermediate thiolactone (**6**) solely through CYP1A2-, CYP3A4-, CYP2C9- and CYP2C19-dependent transformations.

The drug disposition of clopidogrel is further complicated by the fact that only approximately 15% of the drug is metabolized by the CYP pathway to its bioactive thiol form, with the majority suffering hydrolysis to an inactive acid derivative (**7**). As a result, patients often receive a relatively low exposure of the active form of the drug, which can lead by its very nature to a variable onset of action and response. This is further heightened in patients carrying a CYP2C19 reduced functional allele, in which their ability to metabolically generate the bioactive thiol is further compromised, leading to nonresponsiveness and a higher rate of adverse clinical events. In an acute coronary setting this can take the form of death from cardiovascular causes, myocardial infarction or stroke. In comparison to clopidogrel, the onset of action of prasugrel is rapid and more sustained, and it is less prone to adverse events involving CYP2C19 polymorphism, given a lack of involvement of this CYP in its bioactive thiol generation.

The hepatocyte is well tuned to sensing chemical stress caused by exposure to reactive metabolites. With respect to the molecular mechanisms that come into play, a huge amount of fundamental information has been mapped out from a detailed understanding of events caused by dosing of paracetamol (6). As part of the disposition of paracetamol, a small proportion is converted to the reactive metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI), which is quenched by hepatic glutathione (GSH) or cysteine residues within KEAP1, a cytosolic protein that binds to the redox-sensitive transcription factor Nrf2. Under normal physiological conditions, the role of KEAP1 is to maintain Nrf2 transcription in check through a proteasome-dependent degradation of the protein. This process is disrupted by the covalent binding of NAPQI, with liberation of Nrf2 and subsequent translocation to the nucleus, where it orchestrates an antioxidant response through the activation of cell defense genes, such as glutathione transferases, NAD(P)H quinine oxidoreductase, heme oxygenase and glucuronyltransferase. Unfortunately, at a high (over) dose of paracetamol the extent of NAPQI formation seriously depletes hepatic GSH levels and overwhelms the antioxidant line of defense, resulting in the covalent modification of critical proteins, such as γ-glutamylcysteine synthetase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Ca^{2+}/Mg^{2+}-ATPase, which are

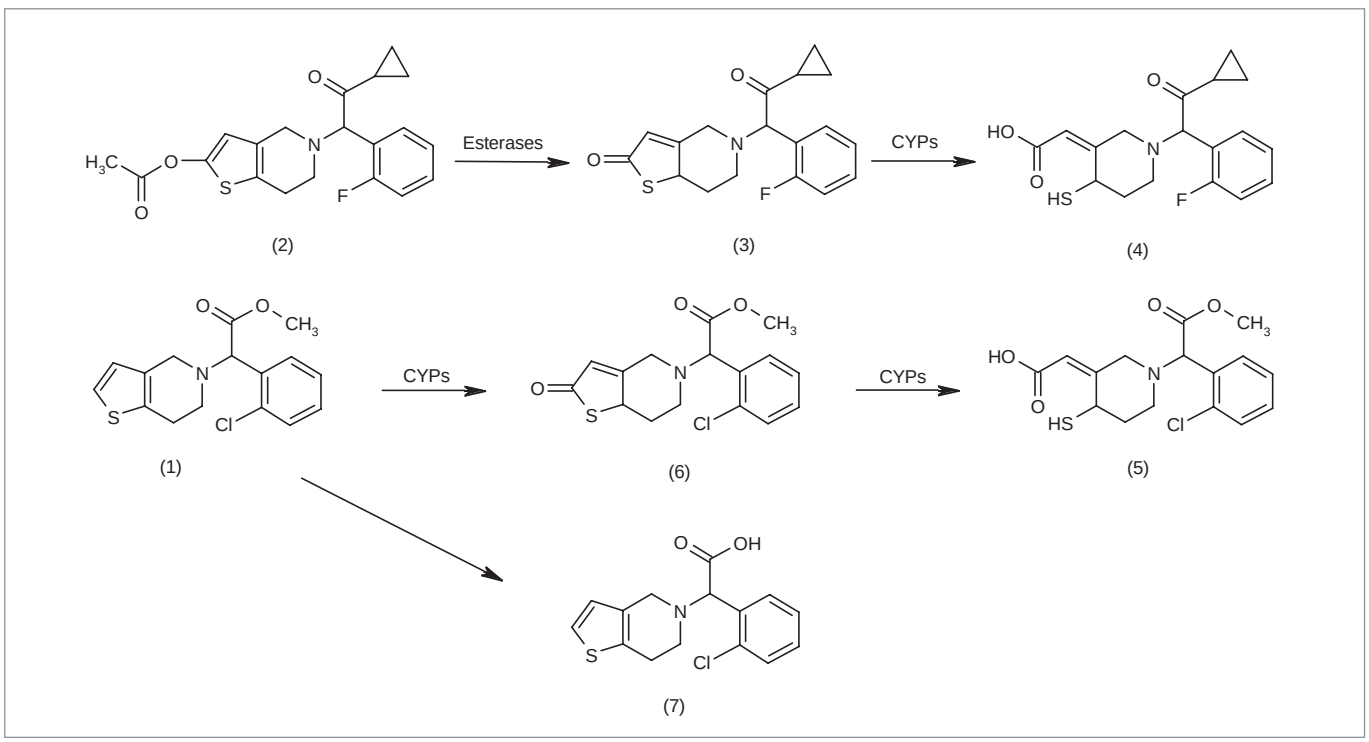

Figure 1. Biotransformation of clopidogrel (**1**) and prasugrel (**2**) to active thiols.

key in maintaining mitochondrial function and Ca^{2+} homeostasis. The impact of such an insult on the cell can lead ultimately to cell death, either by apoptosis or necrosis. Of great interest is that both forms of cell death release specific signature proteins that can be monitored in plasma, namely high-mobility group box protein 1 (HMG-1) from necrotic cells and fragments of cytokeratin-18 (CK-18) from apoptotic cells. From animal studies, increased doses of paracetamol, up to 550 mg/kg, have been correlated with increased plasma levels of these signature proteins, although each one has a unique dose–response profile. Above doses of 550 mg/kg and up to 1000 mg/kg, the levels of CK-18 fragments captured dramatically fall, while in contrast, levels of HMG-1 continue to rise. From the unraveling of the molecular mechanisms of paracetamol toxicity, HMG-1 and CK-18 fragments appear as potential biomarkers for cellular necrosis and apoptosis, respectively. Such relationships have found utility in the diagnosis and severity grading of a number of diseases (7, 8).

Hypersensitivity is a common adverse event with many drugs that can limit their therapeutic use; examples include carbamazepine, sulfamethoxazole, abacavir and nevirapine. There is a growing body of evidence that reactive drug metabolites may play a key role in eliciting the allergic reaction, which is believed to center on activation of the T-lymphocyte system (9). Although a lymphocyte transformation test (10) is available as an in vitro test to assess the propensity of a drug or its metabolite to cause hypersensitivity, further research has revealed a genetic basis that may offer pharmacogenetic screening as a means of de-risking patient drug hypersensitivity. In several clinical studies of abacavir, a nucleoside reverse transcriptase inhibitor designed to combat HIV, avoiding inclusion of patients with the major histocompatibility complex (MHC) class I allele HLA-B*5701 delivered a significantly reduced diagnosis of hypersensitivity (11). Such results represent a potentially important step in the clinical management of abacavir, given that patients presenting hypersensitivity manifest conditions of fever, rash, gastrointestinal and respiratory symptoms that can become more severe, rapid and life-threatening if discontinuation of the drug is not immediately instigated. The HLA-B*5701 genotype has also been advocated as a high risk factor for the drug-induced liver injury caused by flucloxacillin, and prescreening of patients, as in the case of abacavir, may provide enhanced patient compliance (12).

Mr. Andy Harrell (GSK, U.K.) presented a current industry perspective on reactive metabolites. Since their identification in the 1930s, there has been great interest in the area. During the last 30 years of the 20th century, the association of reactive metabolites with toxicity and withdrawal of certain classes of drugs, such as β-lactam antibiotics (anaphylaxis), nonsteroidal anti-inflammatory drugs (NSAIDs; idiosyncratic hepatoxicity) and arylamines (carcinogenicity and agranulocytosis), began to emerge. Several hypotheses (hapten and critical protein) were proposed to support the link at a mechanistic level, in which a central tenet focused on covalent binding of the reactive metabolite to proteins or DNA. As a consequence, during the last 10 years a multitude of nonradioactive reactive metabolite screens have become engrained within drug discovery in an attempt to identify development candidates where risk from reactive metabolite generation and toxicity is minimized. However, during the early period of this paradigm shift to reactive metabolite screening, little scientific data existed in the public domain to support decision-making.

To fill this void, GSK initiated an internal program aimed at defining certain guidelines. In addition, it was hoped that such an output could help address the high proportion of compounds failing to progress within the organization due to unacceptable hepatoxicity. To this end, a database was established consisting of 200 hepatotoxic and 200 nonhepatotoxic known drugs. Criteria for entry into the hepatotoxic class of drug consisted of at least 50 reports of hepatotoxicity or at least 3 reports of life-threatening hepatotoxicity, warnings or precautions in label, including liver enzyme monitoring. In contrast, no reports of hepatotoxicity or only one report of "mild" hepatoxicity constituted the nonhepatotoxic class of drugs.

Initially, two methodologies, often used in lead optimization, were used to assess the propensity for reactive metabolite formation. The first centered on GSH trapping, where production of GSH conjugates from microsomal incubations was monitored via loss of pyroglutamic acid (m/z 129). Using a signal:noise ratio approach, the extent of GSH conjugative production could be quantified (ratios: minor 1-10; notable 10-100; and marked > 100). The second assay focused on CYP time-dependent inhibition (TDI). The CYPs employed covered 1A2, 2C9, 2C19, 2D6, 3A4DEF and 3A47BQ, and the extent of the IC_{50} fold change with time allowed a ranking distribution of the drugs into subsets (fold shift: minor < 2; notable 2-5; and marked > 5). Results from the GSH trapping screen revealed that 80% of GSH adducts classified as marked adhered to the hepatotoxic class of drugs. In the case of marked TDI, this figure dropped to 70%. However, common to both screens was a cluster of drugs displaying marked GSH/TDI effects but not exhibiting hepatotoxicity. Further investigation of this cluster revealed that many of the drugs were administered as a low dose (< 10 mg) or intermittently or topically applied. Revisiting the analysis and setting the drug dose threshold to > 100 mg/day delivered an improved discrimination. Of those drugs that exhibited hepatotoxicity in man, 96% and 82%, respectively, gave marked GSH adducts and TDI effects.

The availability of radiolabeled drugs allows a further assessment of the extent of drug protein adducts and the risk of toxicity, in particular idiosyncratic toxicity (IDT), through bioactivation mechanisms. Covalent binding measurements can be performed in liver microsomal or hepatocyte preparations from both animals and humans. In the case of animals, the level of adducts to both liver and plasma proteins can be determined in the intact animal after oral dosing. Seminal work by the Merck group (13) suggested 50 pmol drug eq/mg protein as a target upper limit, above which a compound would not be advanced into development unless other qualifying considerations were taken into account, such as a daily dose < 10 mg or if the disease under treatment was life-threatening.

As part of the GSK predictive toxicology initiative, 65 radiolabeled drugs of known safety profiles were screened in a microsomal activation assay, in which residual radioactivity associated with unextracted protein was measured. Analysis of the results suggested 200 pmol drug eq/mg of protein as an alert. In a second GSK study, analysis of ex vivo covalent binding data, easily generated from low-dose toxicology studies, suggested that > 30% unrecovered radioactivity at a concentration of > 1 pmol represented a further risk alert.

Reference was also made to recently published work by researchers at Daiichi Sankyo (14), who screened 42 radiolabeled drugs of known safety profiles for covalent binding in human microsomes, human

hepatocytes and rat liver in vivo. They also concluded that 50 pmol drug eq/mg of protein was ineffective in grouping the safety categories, with drugs given a warming of IDT in the Physician Desk Reference and Japanese drug labeling not exceeding this threshold, and 4 of the 12 so-called safe drugs exhibiting covalent binding in human microsomes that exceeded 100 pmol drug eq/mg of protein. Indeed, the use of covalent binding alone in each of the three test systems failed to distinguish the safety categories. Only when clinical daily dose was taken into account did a significant distribution plot become clear, with the separation of drugs into safe, equivocal and dangerous zones.

In concluding the talk, Mr. Harrell suggested there was a move away from blanket screening for reactive metabolites to a more bespoke application of various screening models that could be applied at different stages of a project. Risk assessment guidelines were now available from such models, with several incorporating a clinical dose input that led to a higher level of predictivity. As an underlying theme, increased dose appeared to be a clear driver for increased risk.

Professor Julien Blagg (Cancer Research U.K. Centre for Cancer Therapeutics) delivered a talk on the role of medicinal chemistry design in the avoidance of toxicity at clinically effective exposures. In setting the scene, clinical attrition drivers over a 10-year period spanning 1991-2000 were highlighted (15). In contrast to a dramatic decline in attrition through pharmacokinetic aspects, the most prominent cause in 1991 to a minor bit player in 2000, drug failure due to toxicology almost doubled to become a major factor entering the 21st century. In addition a slight increase was also observed due to lack of clinical safety. However, Professor Blagg suggested preclinical attrition rates due to safety and toxicity findings were likely to be significantly higher, given that such data often resided in confidential corporate archives.

Although mechanistically adverse outcomes could be linked to primary pharmacology, secondary pharmacology or the presence of a structural alert or toxicophore, meta-analysis of in vivo tolerance (IVT) studies conducted on a dataset comprised of 245 potential Pfizer drug candidates accumulated over a 5-year period support a further origin in the form of physicochemical drug properties (16). For each IVT study, the corresponding pharmacokinetic exposure data were also collected (C_{max} and AUC).

Cross-comparison of the chemical space property distribution map (MW, CLogP, TPSA) of the IVT compound dataset with that of a diverse subset of the Pfizer corporate file showed good overlap, thereby confirming that the set of compounds occupied sufficient chemical diversity and the results of the meta-analysis hold general application. Of the dataset, 50% were of a basic nature, 40% neutral and 10% acidic.

Key to the meta-analysis was the importance of data deconvolution, and at what specific exposure threshold should intrinsic toxicity be judged. C_{max} was used as the single parameter to reflect exposure, and 10 μM total drug was selected as a pragmatic threshold level that delivered a distribution of compounds between toxic and clean, while at the same time minimizing the number of compounds classified as uncertain. Data associated with compounds in which the adverse in vivo outcome could correlate with the primary pharmacology of the compound were removed from the analysis. Defined lists of significant toxicology markers, generated from histopathology, clinical pathology or clinical signs, were used to label the compounds. Free drug exposure could be substituted for total drug exposure, providing plasma protein binding data were available. Unfortunately, due to a lack of historical data, only 72% of the original dataset met this criteria. However, by following a similar line of data deconvolution, the appropriate free drug C_{max} threshold was determined to be 1 μM. Regardless of whether free or total drug C_{max} was examined, analysis of the drug toxicity classification using a wide range of physicochemical properties and descriptors revealed a consistent link with both TPSA and CLogP. Calculation of toxicity odds ratios revealed an inter-relationship between these properties, and by setting cut-offs for CLogP and TPSA of 3 and 75 A^2, respectively, identified compounds with CLogP > 3 and TPSA < 75 A^2 as being six times more likely to have an impactful toxic outcome than compounds with CLogP < 3 and TPSA > 75 A^2, whether based on total or free drug exposure. Compounds exhibiting only a single risk factor gave a weak and inconsistent trend. The addition of a further 2 years of new IVT data reinforced the high CLogP (> 3)/low TPSA (< 75 A^2) trend, with an increase in the odds ratio to 10-fold using total drug C_{max} as a reference point. Substitution to a free drug analysis delivered a further rise to 27-fold.

As a hypothesis, it was suggested that promiscuous binding to off-target pharmacology was responsible for the increased incidence of adverse outcomes associated with high CLogP/low TPSA space. To support the theory, CEREP data across the Bioprint™ panel of 48 assays of varied target class (G protein-coupled receptors [GPCRs], enzymes, ion channels) on 108 compounds were analyzed, using > 50% inhibition at 10 μM at three or more targets as a definition of promiscuity. In line with the observations derived at a toxicity level, compounds of CLogP > 3 and TPSA < 75 A^2 appeared 25 times more likely to have a significant off-target pharmacological profile than compounds of CLogP < 3 and TPSA > 75 A^2. High lipophilicity has often been a design parameter used by the medicinal chemist in driving high potency against a target. Given its link to imparting an increased risk of toxicity and promiscuity, as well as high clearance, Pfizer researchers have coined the term LipE, as defined by equation (1), as a lipophilicity efficiency measurement that can be used by the medicinal chemist to ensure increases in potency and compound design are directed away from liphophilicity risk factors associated with toxicity and promiscuity (17).

$$\text{LipE} = \text{p}IC_{50} \text{ (or p}EC_{50}) - \text{CLogP} \quad \text{(Eq. 1)}$$

In relation to idiosyncratic adverse events triggered by chemical structure, in particular through metabolic activation, Professor Blagg focused on reducing dose size as a means of preventing attrition through this mechanism. The closely related analogues clozapine (**8**) and olanzapine (**9**) served to illustrate the case (Fig. 2). Clozapine, at a clinical dose of 300 mg/day, is known to form reactive metabolites in vitro and cause a 1% incidence of argranulocytosis. In contrast to the restricted use of clozapine, olanzapine, dosed at 10 mg/day, shows no incidence of agranulocytosis, despite in vitro studies confirming reactive metabolite formation.

Furthermore, documented work has shown that drugs dosed at < 10 mg/day were associated with a significantly lower incidence of idiosyncratic drug reactions (18). Notwithstanding imparting the desired

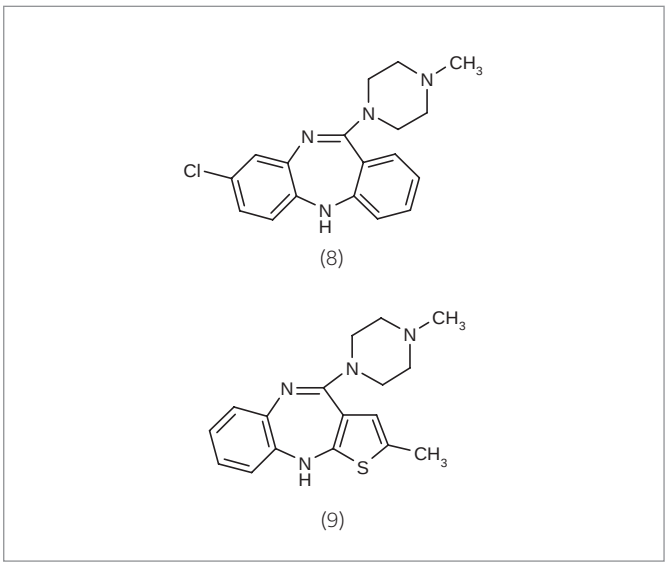

Figure 2. Structures of clozapine (**8**) and olanzapine (**9**).

intrinsic potency at the target, such a low projected clinical dose demands in conjunction superb pharmacokinetic and physicochemical properties, all of which remain under the remit and influence of medicinal chemistry design.

The afternoon session of the symposium was opened by Dr. Martin Traebert (Head of Safety Pharmacology EU, Novartis Institute for Biomedical Research) with a presentation entitled "Addressing cardiac sodium channel liabilities during preclinical drug development". Dr. Traebert began by describing how, historically, pharmaceutical industry interest in cardiac sodium channels had centered on trying to develop inhibitors as antiarrhythmic agents. Encainide and flecainide were two drugs progressed into clinical development and investigated in the Cardiac Arrythmia Suppression Trial (CAST). Unfortunately, however, these drugs were shown to cause a 3.6-fold increase in arrhythmic death and a 2.5-fold increase in overall mortality (19). Molecular biology studies have revealed that the human cardiac sodium channel contains four transmembrane repeats and is encoded by the gene *SCN5A*. The channel displays strictly voltage-dependent activation, and is responsible for the depolarization phase and "upstroke" of the action potential. Inherited loss-of-function mutations of *SCN5A* are associated with a range of channelopathies, including congenital long Q-T syndrome, idiopathic ventricular fibrillation (Brugada syndrome), isolated cardiac conduction disease, atrial standstill, congenital sick sinus syndrome, sudden infant death syndrome and dilated cardiomyopathy.

Currently, although there is now increased awareness of sodium channel liabilities by regulatory authorities, there are no formal guidelines on this topic. Drug companies are, however, deploying a range of preclinical assays to identify compounds possessing sodium channel liabilities. Novartis routinely uses a binding assay, with rat brain as a sodium channel source, as a screen during lead selection. Moving towards candidate selection, compounds are tested in repolarization assays using the patch clamp technique with HEK-293 or CHO cells stably transfected with *SCN5A*. Compounds are also profiled during candidate selection for cardiovascular effects in vivo using telemetered dogs. Patch clamp assays are clearly more informative than binding assays, but as a manual assay throughput has been limited. Newer, automated patch clamp robots (QPatch) are now available and have markedly increased compound throughput per FTE. A further emerging technique with potential utility to be deployed as part of a screening strategy is compound profiling in spontaneously beating cardiomyocytes derived from human embryonic stem cells. Novartis investigates the effects of candidate compounds on cardiac action potentials in vivo using small animal models. Rabbits and guinea pigs respond similarly to humans in response to sodium channel blockade, displaying prolonged P-R and QRS intervals. During preclinical development, Novartis conducts ECG profiling using telemetry in beagle dogs. Early sighting studies are done using a noninvasive jacket telemetry system, with studies in chronically implanted animals being undertaken prior to phase I.

Dr. Traebert briefly described a Novartis case study demonstrating the utility of the aforementioned battery of assays to identify a compound with clear sodium channel liabilities that was dropped from development. In vivo in dogs the compound affected P-R and QRS intervals, mean P wave duration, and also induced ventricular tachycardia. Tachycardia was also observed in isolated rabbit heart. In vitro the compound was active in cells stably transfected with the *SCN5A* gene. Dr. Traebert concluded by stressing the importance of screening for sodium channel activity during compound development, adding that consideration of this area is underestimated compared to the investigation of compound activity against hERG channels. A variety of in vitro, ex vivo and in vivo assays are available, and significant inhibition that translates into ECG alterations should be a clear "no-go" for drug development.

The second speaker in the afternoon session was Dr. James Dykens (Director of Investigative Cellular Toxicity, Pfizer, U.K.). Dr. Dykens began his presentation by highlighting the problem caused by adverse drug reactions, referencing data from the U.S. Over 2.2 million adverse drug reactions occur annually in hospitalized patients alone in the U.S., leading to approximately 106,000 deaths/year. Adverse drug reactions are the fourth leading cause of death in the U.S. Dr. Dykens view was that serious drug toxicity liabilities were clearly not being identified, and proposed that off-target drug effects on mitochondria were important in this respect. Many drugs withdrawn from the market or receiving black box warnings have been shown to impair mitochondrial function. The pharmaceutical industry is now starting to research this area more closely using newly developed methodologies.

Mitochondria are the "gatekeepers" of cell death. If mitochondria die, then so does the cell. These organelles have evolved from ancient bacteria, and mitochondrial DNA represents the only non-nuclear genome in all animals. Mitochondria generate > 90% of cellular energy, and the magnitude of this activity is highlighted by the fact that human males turn over 193 lbs of ATP/day, while females turn over approximately 148 lbs/day. Studies have shown that drugs can interfere with mitochondrial electron transfer and ATP generation at many points. A Pfizer study of 550 drugs revealed that 34% of drugs displaying organ toxicity impaired mitochondrial function. Drugs impairing mitochondrial function will have an adverse effect,

although clinical manifestation is dependent on the bioenergetic capacity of an individual animal or patient. Bioenergetic capacity is determined by genetics and also age. Indeed, older animals have been shown to be more susceptible to troglitazone-induced toxicity than younger healthy animals.

Dr. Dykens went on to discuss further his experience at Pfizer using a range of assays to detect drugs possessing mitochondrial toxicity. The first assay described was a 96-well plate format mitochondrial respiration assay (20, 21). This assay revealed potent inhibitory activity for a number of thiazolidinediones, and subsequent work with a pioglitazone photoaffinity probe pulled down MitoNEET, an atypical 2Fe-2S protein, as a molecular target for this drug. A further mitochondrial functional assay utilizes "Seahorse technology". This system measures oxygen consumption rate and extracellular acidification rate in microchambers. Phenformin and butformin, drugs withdrawn for causing lactic acidosis, were shown to be active in this assay, while metformin was inactive (22). Mitochondrial impairment by thiazolidinediones and statins was also demonstrated using an assay format that measures the activity of individual oxidative phosphorylation complexes following immunocapture (23).

Contemporary cell culture conditions typically contain glucose concentrations five times the physiological levels. Elevated glucose inhibits mitochondrial respiration (Crabtree effect), and consequently, drugs possessing mitochondrial toxicity will not be detected under such conditions. However, cells grown in galactose do not generate ATP through glycolysis and become susceptible to drugs inhibiting mitochondrial function (24). In terms of useful in vivo models, heterozygous superoxide dismutase 2 ($SOD^{+/-}$) mice display clinically silent mitochondrial dysfunction, and in contrast to normal mice are susceptible to the hepatotoxic effects of troglitazone (25). Dr. Dykens concluded his talk by reiterating his view that a key contributor to idiosyncratic drug responses is mitochondrial dysfunction. Individual susceptibility is determined by an individual's "bioenergetic threshold". Pfizer has moved assessment of mitochondrial toxicity to the lead selection phase of discovery, where there are potentially diverse hit series and the greatest opportunity to "de-risk" for hepatotoxicity, nephrotoxicity and neurotoxicity.

The next speaker was Dr. Willem Schoonen (Department of Toxicology and Drug Disposition, Schering-Plough, the Netherlands), who presented his company's approach to "High-Throughput Screening for Toxicity Testing" in early drug discovery. Despite the efforts by the pharmaceutical industry over the last 15 years, some 50% of new chemical entities (NCEs) still fail due to toxicity, which represents a major cost to industry (15), particularly in the areas of hepatotoxicity, cardiotoxicity, skin toxicity, CNS side effects, genotoxicity and carcinogenicity. The preclinical cost is even more significant, as safety screening is often the final hurdle in drug discovery before the NCE enters the clinic, and few effective strategies for avoiding toxicity exist to guide medicinal chemistry programs before then (26, 27).

Dr. Schoonen described the assays Schering-Plough uses to assess DNA and membrane damage, cell-, organ- or organelle-specific toxicity, nuclear receptor activation and CYP enzymes and induction, which are used to select/deselect and/or rank compounds in lead optimization. What assay in which phase depends on sample throughput, the amount of compound available and the effort required in conducting the assay. Multiple leads are screened and multiple measures of in vitro toxicity are used to aid compound optimization in order to reduce the attrition rate in the preclinical and clinical phases, as detailed below.

–Genotoxicity	Vitotox, p53-RE
–Clastogenicity	RadarScreen, p53-RE RAD51C Cystatin A
–Carcinogenicity	RadarScreen, p53-RE
–Nongenotoxic carcinogenicity	Human and rat AhR, rat PPARα
–Cytotoxicity	Glu, Calcein, Alamar Blue, Hoechst Luxcel
–Nuclear receptors	PXR, PPARγ, FXR, LXR, CAR
–Cytochrome P450 luciferase	1A1, 1A2, 2C8, 2C9, 3A4 (Promega assays)
–Cytochrome P450 enzymes	1A1, 1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4
Throughput: 40-80 compounds per assay batch	

For fast genotoxicity screening, a panel of six screens is used, all of which depend on sensitive luminescent luciferase-based assays. A bacterial screen is performed in *Salmonella*, where the luciferase expression is activated via a cascade of reactions known as the SOS system, which is employed in the "Vitotox" assay to detect the genotoxicity, cytotoxicity and mutagenic potency of the sample (Fig. 3).

The major benefit of the assay is that the entire DNA content of the cell functions as a target for the genotoxin to display its effect, and can be considered as a substitute for the full Ames mutagencity test. With respect to clastogenicity, a "RadarScreen" (reMYND, Leuven, Belgium) assay is used to replace the sister chromatid exchange (SCE), chromosomal aberration (CA) assays and micronuclei tests, which are time-consuming and have low compound throughput. The assay is based on activation of the RAD54 promoter linked to a β-galactosidase reporter gene in yeast, as shown below (Fig. 4).

Good sensitivity, selectivity and predictability for the clastogenicity test are observed, although prediction for mutagenicity is relatively low, which is the opposite for the Vitotox assay. More mechanistic assays for genotoxicity in Hep G2 cells were described, in which four promoters of the luciferase assay were used. An advantage of these cells is their ability to metabolize/activate certain drugs such as benzo[*a*]pyrene, aflaxtoxin B1 and etoposide, without the need for the S9 metabolic activating system (28-30). Although the cells do not have CYP2C9 activity, it can be induced.

All of these assays were validated against a large number of genotoxic (and nongenotoxic) compounds (190) with diverse mechanisms, including direct-acting genotoxins, topoisomerase inhibitors, nucleotide/DNA synthesis inhibitors, reactive oxygen species generators and aneugenes (change in number of chromosomes). In general, the sensitivity, specificity and predictivity of the assays are acceptable, with a total of 110 compounds having a positive Ames or clastogenicity score (31). It was concluded that Vitotox prediction is relatively high, RadarScreen is very good and prediction of all four different Hep G2 assays is relatively low in comparison with *Salmonella* and yeast genotoxicity assays. Nevertheless, in vitro high-throughput screening might be more valuable for the prediction of human genotoxicity (32, 33).

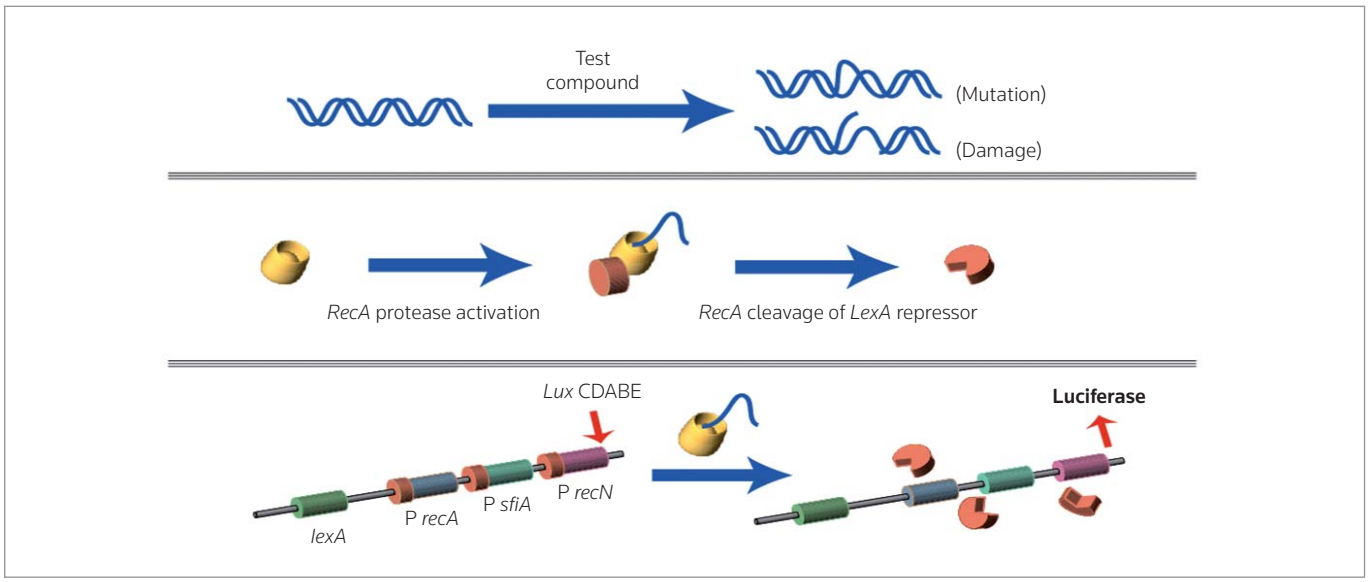

Figure 3. Vitotox assay principle.

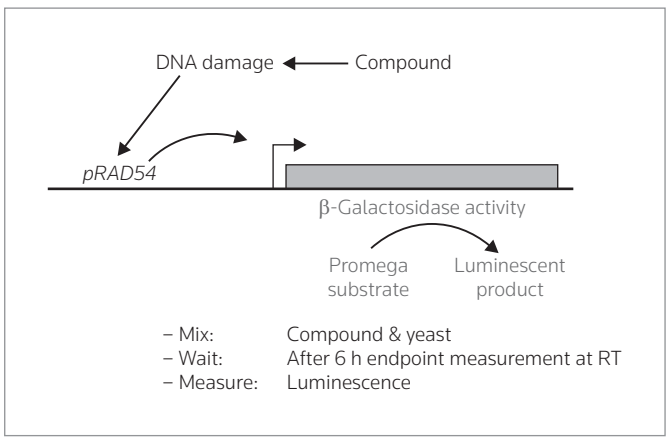

Figure 4. RadarScreen assay principle.

The last speaker of the day was Dr. Adrian Hill (Evotec, U.K.), who gave a presentation on the use of zebrafish screening in early drug discovery, with reference in particular to predicting hepatotoxicity, cardiotoxicity and developmental toxicity (embryotoxicity and teratogenicity). Although zebrafish have been used as an experimental tool for the last 40 years, more recently they have been used as an in vivo vertebrate model to support early drug discovery for screening NCEs. The small size of the larvae makes them amenable to high-throughput screening using very small quantities of compound. Their metabolism, physiology and development are apparently comparable to humans, and since they are transparent, multiple endpoints can be visualized without dissection. Dr. Hill went on to describe several assays that have been developed in conjunction with a number of pharmaceutical companies, as detailed below (Table I).

Uptake into zebrafish can vary considerably from compound to compound, and no one physicochemical property can adequately predict compound uptake through the skin. A generic protocol is therefore employed to measure the uptake of compounds using conventional bioanalytical methods, which helps to identify false negatives and to correlate the extent of exposure with toxicity.

Data were presented on a cardiac functional assay, where it was concluded that the cardiophysiological response of zebrafish was predictive of the human cardiovascular response. Some 64 compounds were used in the validation, a summary of which is presented below:

Table I. *Multifunctional uses of zebrafish in drug discovery.*

Toxicology	Safety pharmacology	Disease models
Hepatotoxicity	Cardiac safety	Epilepsy
Developmental toxicity (embryotoxicity & teratogenicity)	Gut motility Visual function	Nonassociative learning Cartilage + bone staining
Acute toxicity	Auditory function	Ototoxicity
Myelotoxicity	Locomotor activity	

–Atrioventricular decoupling: 31 of 36 (86%)
  Two causing no cardiac effect (erythromycin, sotalol) – poor uptake
  Three causing bradycardia (mibefradil, nortriptyline, ranolazine)
–Bradycardia: 11 of 12
  One causing atrioventricular decoupling (diltiazem)
–Tachycardia: 8 of 8
–Negative controls: 8 of 8
–Sensitivity = 89%, specificity = 100%
–Overall predictivity = 91% (excluding bioanalysis data)

Information was also presented from a Novartis (blinded) study using 20 compounds, in which bradycardia was correctly predicted for 5 of 5 compounds. In terms of Q-T-prolonging drugs: 91% were correctly identified by atrioventricular decoupling (reducing to 71% due to false negatives with poor uptake). Excluding unclassified compounds, the overall predictivity amounted to 94%.

In terms of developmental toxicity (embryotoxicity and teratogenicity), a number of institutions have used a zebrafish assay employing different protocols and strains/ages of larvae. Endpoints associated with teratogenicity are detailed in Table II. Overall predictivity remained high, between 80% and 95%.

Finally, in terms of hepatotoxicity, a zebrafish assay was described using a number of endpoints, including liver abnormalities, changes in size and shape of the liver (hepatomegaly), effects on yolk absorption (yolk retention), lethality, gastrointestinal toxicity and loss of bile. The assay was compared to a novel cell-based model using a high-content screening (HCS) model (34). In addition to 14 well-known toxic compounds, a subset of 36 compounds from the HCS assay was selected for screening including:

–Human hepatotoxic compounds that gave a false negative in the HCS (stavudine, novobiocin, bupropion, diethylcarbamazine, clofibrate, pravastatin, valproate)

–Toxic compounds to animals that gave a false negative in the HCS (ridogrel, acaftadine)

–Toxic compounds to humans but NOT liver toxic that gave a false positive in the HCS (metformin [pancreas], pamidronate [kidney], astemizole [acute], temozolomide [bone marrow], gentamicin)

–Nontoxic compounds: false positive in the HCS (picotamide)

The HCS resulted in 6 of 9 false positives (gentamicin, metformin, astemizole, temozolomide, picotamide, pamidronate) and 9 of 27 false negatives (clofibrate, stavudine, novobiocin, bupropion, diethylcarbamazine, pravastatin, ridogrel, acaftadine, valproate). Overall, the HCS assay sensitivity was 67%, specificity 33% and predictivity 58%. In comparison, the zebrafish assay gave 3 of 50 false positives (gentamicin, praziquantel, astemizole) and 5 of 50 false negatives (oxyphenisatin, valproate, ketoconazole, novobiocin, stavudine), leading to an increased sensitivity of 86%, specificity of 77% and overall predictivity of 84%.

Table II. *Zebrafish endpoints associated with teratogenicicity.*

Development	Retardation/arrest
Body shape	Abnormal body shape/short body/tail
Eyes	Eye abnormalities
Ears	Ear/otolith abnormalities
CNS	Brain/spinal cord abnormalities
Cardiovascular	Heart morphology/anemia/angiogenesis
Pigmentation	Pigment abnormalities
Jaws/arches	Jaw abnormalities
Fins/skin	Dorsal/caudal fin abnormalities Pectoral fin abnormalities

DISCLOSURES

J. Allen, P. Jeffrey and A. Ratcliffe are in the paid employ of their respective companies. All authors are SMR Committee members for which no remuneration is paid.

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