The Need for Revision of Pre-Market Testing

The Failure of Animal Tests of COX-2 Inhibitors

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FDA Open Public Hearing

Arthritis Advisory Committee
Drug Safety and Risk Management Advisory Committee

February 17, 2005

PCRM
Physicians Committee for Responsible Medicine
5100 Wisconsin Ave. NW, Suite 400 • Washington, D.C. 20016
202-686-2210 • www.PCRM.org

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Summary Statement

The recent withdrawal of Vioxx and the growing concern over the safety of other COX-2 inhibitors have promoted a re-evaluation of our nation's system of drug development, approval, marketing, and monitoring. But one critical factor must be addressed: the distorting effect of animal tests on the evaluation of the safety of not only COX-2 inhibitors, but of other pharmaceuticals as well.

Animal tests have often proven to be misleading and potentially dangerous for the evaluation of drugs that will be prescribed for humans. Reasons include significant and immutable differences among and within animal species (including humans) regarding anatomy, physiology and drug metabolism. These differences result from genetic diversity and have become better understood and characterized by new information and technologies arising from the Human Genome Project. The use of genetically modified research animals, nonphysiological approaches attempting to duplicate human diseases, and data derived from physiologically altered animals due to unavoidable stress in the laboratory environment raise further complications in interpretation.

Although it is not possible with current technology to identify all possible drug risks completely until many patients have been exposed over long periods, it is very possible to evaluate candidate drugs more accurately by replacing animal studies with superior evaluation methods. These methods include appropriate use of epidemiological data, improved human pharmacological assessment (such as with microdosing studies), incorporation of sophisticated *in vitro* and *in silico* processes, use of recombinant DNA technology, microarrays (cell protein and DNA), and incorporation of the transforming tools available from stem cell techniques and pharmacogenomics.

This paper presents a review of the COX-2 controversy, including specific information regarding the misleading and harmful role played by animal tests during all aspects of pre-approval testing. Explanations for the unsuitability of animal research in drug development are presented, and the superiority of replacement methods is reviewed. Finally, the paper makes several recommendations for improving upon current drug development and approval processes. Note, however, that these or any other corrective measures adopted in these processes, will be seriously inadequate unless the focus is shifted completely and specifically on the species at risk: humans.

I. Review of the Vioxx Controversy

On September 30, 2004, Merck and Co., Inc., withdrew its blockbuster drug Vioxx (rofecoxib) from world markets. One of three cyclooxygenase-2 (COX-2) inhibitors approved by the U.S. Food and Drug Administration (FDA), Vioxx was marketed in over 80 countries and had worldwide sales of over $2.5 billion in 2003. Merck's action followed the report of early termination of the Adenomatous Polyp Prevention on Vioxx (APPROVe) clinical trial due to excess risk for heart attack and stroke in subjects taking 25 mg of Vioxx daily. The study included 2,600 subjects and was designed to evaluate Vioxx's benefit in decreasing recurrence of colon polyps. The Merck-funded study reported 1.48% cardiovascular (CV) event risk for subjects taking Vioxx, compared to 0.75% risk for subjects taking a placebo, but these risks were adjusted to 3.5% and 1.9%, respectively, after FDA reviewers corrected improper reporting of clinical events in the study data.

The doubled risk for heart attack and stroke in APPROVe provided irrefutable validation of previous data indicating increased cardiac and vascular event risks for patients taking Vioxx. The Vioxx Gastrointestinal Outcomes Research (VIGOR) study, also funded by Merck, was published in November 2000 (*1*). VIGOR demonstrated a significant advantage for rofecoxib compared to naproxen for decreasing upper gastrointestinal (GI) events, but also identified five times the risk for heart attack among study subjects receiving rofecoxib. The authors proposed, without direct evidence, that this difference was due to a protective effect from naproxen. Unconvinced, the FDA issued a warning letter to Merck president and CEO Raymond Gilmartin on September 17, 2001, stating that Merck had "engaged in a promotional campaign for Vioxx that minimizes the potentially serious cardiovascular findings that were observed in the Vioxx Gastrointestinal Outcomes Research study, and thus, misrepresents the safety profile for Vioxx" (*2*). This letter came seven months after the FDA's Arthritis Advisory Committee expressed concern about the increased CV risk reported in the VIGOR study and one month after a critical review based partly on the information available from that meeting (*3*). Despite recommendations from that report, and from other researchers and physicians, the FDA did not require a label change or additional clinical data regarding Vioxx's safety.

The possibility of a protective effect for naproxen was promoted by a series of case-control studies, two authored by physicians with drug company support (*4,5*), and one written by Merck Research Laboratories employees (*6*). These studies were criticized because of the inherent risks for bias and confounding in case-control studies, because the reported results could not explain the risk difference between rofecoxib and naproxen in VIGOR, and because several much larger cohort studies demonstrated that naproxen provides little or no protective benefit for cardiovascular events (*7–12*).

Konstam's meta-analysis of 23 phase IIb through V rofecoxib clinical trials identified no increased CV event risk for rofecoxib, and described differences between rofecoxib and naproxen as "likely the result of the antiplatelet effects of the latter agent" (*13*). Konstam's report was done on behalf of Merck Research Laboratories. He and a co-author were paid consultants to Merck; the other five authors were employees of Merck Research Laboratories. This report was also criticized because it was underpowered to assess CV risk, and because it reflected low-risk populations typically used in pre-approval drug studies (*14*).

In a meta-analysis of 18 randomized controlled trials and 11 observational studies involving rofecoxib, Juni and colleagues identified a 2.24–2.30 relative risk for heart attack among rofecoxib patients (*9*). Relative risk was consistent whether rofecoxib was compared to a placebo, naproxen, or another nonsteroidal anti-inflammatory drug (NSAID). Juni's analysis also demonstrated that there was little if any protective effect from naproxen, and that the increased risk from rofecoxib was evident as early as the VIGOR study. He concluded that Vioxx "should have been withdrawn several years earlier."

In fact, internal Merck emails and marketing materials show that the company was aware of increased CV risk for Vioxx not only as far back as the VIGOR study in 2000, but as early as 1996—three years before FDA approval. Merck scientists and executives knew that increased CV events were likely with Vioxx unless patients were also allowed to take low-dose aspirin, but that doing so would likely negate the GI toxicity advantage for Vioxx. Merck appears to have attempted to circumvent this issue by limiting clinical evaluations to low-risk patients, promoting alternative explanations for event rate differences, using misleading presentations to doctors, and training its sales staff to "dodge" the CV risk issue (*15*).

When Merck officials met in May 2000 to review the VIGOR data and to consider whether to conduct a clinical trial to evaluate CV risk, they decided not to do so for logistical and marketing reasons. A slide prepared for the meeting stated: "At present, there is no compelling marketing need for such a study. Data would not be available during the critical period. The implied message would not be favorable" (*16*).

The FDA was also aware of potential CV risk from Vioxx at the time of approval, stating in its medical officer review dated May 20, 1999: "The data seem to suggest that…thromboembolic events are more frequent in patients receiving rofecoxib than placebo" (*17*). Based on APPROVe data, there were 16 excess heart attacks or strokes per 1,000 patients studied, projecting to potentially 160,000 excess events for the estimated ten million patients currently exposed to Vioxx (*18*). More than 80 million patients received Vioxx between its FDA approval in May 1999 and its withdrawal in September 2004. Dr. David Graham of the FDA's drug safety office has stated that between 88,000 and 139,000 people have had heart attacks (30–40% of which were fatal) that may be linked to rofecoxib (*19*). Graham also presented an FDA-funded study of 1.4 million patients in the Kaiser Permanente HMO, comparing risks for heart attack and sudden cardiac death among patients receiving rofecoxib, celecoxib (another COX-2 inhibitor), and five other NSAIDs (*20*). The presentation at an international meeting in Bordeaux, France, in August 2004 reported tripled CV event risk for rofecoxib patients receiving more than 25 mg per day, compared to risk-matched controls. Even at doses less than or equal to 25 mg per day, rofecoxib had a 50% greater event risk than celecoxib.

The CV risks of rofecoxib are now proved, and the efforts of Merck to conceal, minimize, or obfuscate those risks have been exposed. One editorialist characterized the actions of Merck and the FDA as "ruthless, shortsighted, and irresponsible self-interest" (*21*). The details of Merck and FDA responses to accumulating evidence since 1996 are provocative and are being investigated by Congress. Merck faces liability risks estimated at $10–38 billion. But what of the other approved COX-2 inhibitors, celecoxib (Celebrex) and valdecoxib (Bextra), both Pfizer drugs? Are their risks similar to that of rofecoxib, and is there evidence that increased CV event risk is a class effect?

II. The Controversy Spreads: Celebrex and Bextra

Immediately upon the demise of Merck's Vioxx, Pfizer, Inc., hurried to tout the safety of its COX-2 inhibitors, Celebrex and Bextra. Enormous market gains were at stake, and Pfizer stood to receive them all. Adding any substantial portion of the estimated 14 million Vioxx prescriptions and $2.5 billion in sales in 2004 to the estimated 20 million prescriptions for Celebrex and 11 million for Bextra would be a windfall. Intensive direct-to-consumer advertising was undertaken, including direct mail, print and television ads, and a 27-minute infomercial titled "On the Road to Joint Pain Relief."

Pfizer promoted the safety and effectiveness of Celebrex largely based upon the results of the Celecoxib Long-term Arthritis Safety Study (CLASS), a double-blind randomized controlled study comparing gastrointestinal toxicity among celecoxib, ibuprofen, and diclofenac (*22*). Study authors concluded that celecoxib showed less GI toxicity than the two standard NSAIDs, but this conclusion was refuted by subsequent reviews of the study design and endpoints (*23,24*). It is noteworthy that CLASS was sponsored by Pharmacia, manufacturer of Celebrex, and that all 16 authors (including faculty from eight medical schools) were either Pharmacia employees or paid consultants to the company (*25*).

As published, CLASS was substantially altered compared to the initial study design submitted to the FDA. The results reported actually referred to a combined analysis of the first six months of two separate and longer trials, intended to compare celecoxib individually to ibuprofen

and diclofenac (*23–25*). The durations of the original two trials were 12 and 15 months, respectively, rather than the six-month duration reported in CLASS. Data reporting was limited to six months because of a gradually increasing dropout rate for all drug groups during the remainder of the studies, a ploy that was criticized during a subsequent FDA briefing (*26*) and independent data review (*23*). Additionally, the predetermined primary study endpoint was ulcer-related complications and did not include the softer endpoint of "symptomatic upper GI ulcers" reported in CLASS (*23*).

Explicit statistical comparisons in the original protocol were altered, which, in combination with the expanded endpoint definition and shortened follow-up, allowed the authors to conclude that celecoxib was superior to ibuprofen and diclofenac in preventing GI complications. FDA review and data analysis concluded that celecoxib showed no such benefit whether compared to either NSAID alone or to the combined results for ibuprofen and diclofenac (*26,27*). One reviewer also listed as his first conclusion: "Celecoxib does not appear to be more effective for treating the signs and symptoms of OA or RA than the NSAID comparators" (*27*). The complete CLASS data therefore showed neither superior efficacy nor superior safety for celecoxib.

In order to show GI toxicity advantage for celecoxib, Pharmacia (acquired by Pfizer in April 2003) reported data from a post hoc analysis of study subjects not using aspirin. This approach was also rejected by the FDA reviewer (*26*), and, in fact, a similar retrospective analysis also demonstrated a higher CV event risk for celecoxib users not using aspirin (*28*). Pharmacia also sponsored a post hoc analysis demonstrating no increased CV event risk when all CLASS subjects were included (*29*). As with Vioxx, analysis of the CLASS data indicates that COX-2 inhibitors may show *either* decreased GI toxicity *or* no increased CV risk, but not both. Study population risk profile and concomitant aspirin use appear to be the determinants. Subsequent attempts to rationalize the manipulation of data in CLASS (*30*) were rebuked (*23*). The discrepancies between CLASS as submitted for publication to JAMA and as eventually reviewed by the FDA contributed to adoption of a JAMA policy to require that, for all company-sponsored studies, an independent author take "responsibility for the integrity of the data and the accuracy of the data analyses" (*25*).

JAMA's unintentional publication of flawed data, and the accompanying favorable editorial (*31*), presaged a dramatic increase in worldwide Celebrex sales, from $2.623 billion in 2000 to $3.114 billion in 2001 (*32*). After Merck's withdrawal of Vioxx, Pfizer rushed to produce data indicating that Celebrex did not share Vioxx's CV risk. Mimicking Merck's use of flawed case-control studies by authors with financial support from the company, two reports suggested no increased CV risk for celecoxib when compared to rofecoxib or nonusers of NSAIDs (*33,34*).

On December 17, 2004, it was announced that the National Cancer Institute had prematurely terminated its Adenoma Prevention with Celecoxib clinical trial, due to detection of a 2.5 to 3.4 times increased risk for cardiac death, heart attack, and stroke for celecoxib users compared to placebo. Although another similarly designed trial, Prevention of Spontaneous Adenomatous Polyps, has not shown the same risk at interim data analysis, this additional evidence for an adverse class effect of COX-2 drugs had a chilling effect. Among other measures, National Institutes of Health (NIH) director Elias Zerhouni ordered a safety review of over 40 ongoing NIH-sponsored studies of celecoxib for cancer prevention and treatment, dementia, and other diseases. He also requested "a full review of all NIH-supported studies involving this class of drug" (*35*).

In the wake of this new finding, acting FDA commissioner Lester Crawford noted, "We do have great concern about this product [Celebrex] and this class of products." COX-2 researcher Garret FitzGerald, who had been critical of Merck and the FDA regarding the Vioxx controversy, observed: "I think the trial concludes the controversy about whether there is a class effect of these drugs. Now there is clear evidence of it. You would need to believe the earth is flat if you thought this was just a coincidence" (*36*).

Meanwhile, Bextra (valdecoxib) was experiencing its own set of problems. The drug had been known to produce a rare but potentially fatal skin reaction, Stevens-Johnson Syndrome, since shortly after approval in 2001. Twenty reports of this reaction had been provided to the FDA by November 2002, and the label was changed to identify this risk. Eighty-seven reports of Stevens-Johnson Syndrome and toxic epidermal necrolysis had been filed by November 2004, including 36 hospitalizations and 4 deaths (*37*).

Only two weeks after initiating an intensive advertising campaign for Bextra, Pfizer issued a news release reporting increased CV event risk for valdecoxib, with or without use of its parenteral prodrug parecoxib, in two studies of coronary artery bypass surgery patients. The data reported by Pfizer had previously been excluded from CV risk data submitted to the FDA, but subsequently were reviewed on behalf of the FDA by Dr. Curt Furberg (*38*). One study of 462 patients demonstrated relative CV event risk of 3.40 for parecoxib/valdecoxib patients compared to placebo, when individual events were reviewed (*39*). The second study (1,636 patients) has not been published, but demonstrated relative CV event risk of 2.85 for parecoxib/valdecoxib or valdecoxib alone compared to placebo, despite lower and shorter dosing than in the smaller study.

Analysis of the combined data demonstrated tripled CV event risk for valdecoxib patients (*38*). Furberg concluded, given evidence for such toxicity in coronary artery bypass surgery patients–especially considering the recently demonstrated CV risks for rofecoxib and celecoxib–that " it is prudent to avoid the use of valdecoxib altogether or use it only as a drug of last resort." Pfizer's worldwide medical director for Bextra and Celebrex, Gail Cawkwell, acknowledged the increased risk and stated that "the company cannot ethically test Bextra in patients at high risk for heart disease" (*40*).

In a letter to the *New England Journal of Medicine*, COX-2 researchers Wayne Ray, Marie Griffin, and C. Michael Stein noted that "we write to recommend that clinicians stop prescribing valdecoxib except in extraordinary circumstances…the doubts raised about the safety of valdecoxib constitute a potential imminent hazard to public health and thus require action" (*41*). The FDA criticized Pfizer for withholding Bextra CV risk data, and required label changes including a black box warning for serious skin reactions and a bold type warning contraindicating use in coronary artery bypass surgery (*37*).

Faced with mounting evidence that Pfizer was not forthcoming regarding the true risks for Celebrex and Bextra, that both drugs possessed important CV toxicity, and that Pfizer promoted these drugs in a misleading manner even after toxicity data were reviewed, the FDA issued a warning to the company on January 10, 2005 (*42*). Pfizer was directed to cease specific direct-to-consumer television, print, direct mail, and infomercial ads. The letter stated that these ads omitted material facts (including indications and risks); failed to discuss product labeling; and made misleading statements regarding claims of safety, superiority, and effectiveness. The FDA concluded that the seriousness of the violations "would generally have warranted a Warning Letter; however, in light of your recent agreement to a voluntary suspension on all consumer promotion for Celebrex, we do not feel that it is appropriate at this time" (*42*).

III. Inconsistent Pharmacokinetic and Metabolic Animal Testing

Before any new drug is approved for clinical trials in humans, animal testing is performed to evaluate toxicity, mutagenicity, and teratogenicity. Such testing generally includes evaluations of drug pharmacokinetics (PK), metabolism, and mechanisms of action in at least two animal species. Once phase I and II clinical trials have been performed in humans, it is possible to compare PK, metabolic, and toxicity findings to determine which, if any, animal studies seem to correlate with results in humans. Contrary to popular belief, it is not necessary that study results in any of the animal models be the same as (or even similar to) results in humans in order for more extensive clinical trials and eventual drug approval to occur. As discussed below with regard to the COX-2 inhibitors, animal studies are often inconsistent, species-dependent, and not useful in predicting drug safety or efficacy for humans. It is thus unclear why animal testing is performed.

Numerous animal studies have been performed to evaluate PK and metabolism of COX-2 inhibitors, and comparative findings are available for humans. Rofecoxib has been studied in Sprague-Dawley rats, male beagles, and humans. In rats, rofecoxib absorption was nearly 100% and time to maximum plasma concentration (Tmax) after oral dosing was 0.5 hours (*43*). Rofecoxib demonstrated a unique nonexponential decay of plasma concentration and partial reversible metabolism in rats, due to extensive enterohepatic circulation not seen in dogs or humans (*43,44*). Elimination half-life (t½) and bioavailability for rofecoxib in rats were therefore not determined. Attempts to apply compartmental models to describe rofecoxib PK in rats have failed, due to large intra- and interindividual variations (*45*).

In dogs, absorption after oral dosing was only 36%, Tmax was 1.5 hours, t½ was 3.3 hours, and bioavailability was 26.1% (*43*). Rofecoxib biliary excretion was dominant in rats (68.7%), but not in dogs (26.5%). Terminal urinary and fecal excretions were 25.6% and 72.3% for rats, compared to 17.5% and 76% for dogs. Rofecoxib metabolism was much more complex for dogs than for rats (*43*).

In contrast, absorption after oral dosing of rofecoxib varied with dosage in humans (*46*). Tmax was 9 hours and t½ was 17 hours, both much longer than for either rats or dogs. Bioavailability was nearly 100%. There was virtually no biliary excretion of rofecoxib in humans. Terminal urinary and fecal excretions were 71.5% and 14.2%, inverted compared to rats and dogs. Hydrolysis and reduction were the major metabolic pathways in humans, compared to oxidation in rats and dogs. Highly variable bimodal patterns of rofecoxib concentration-time curves were seen in humans, indicating gene polymorphisms for rofecoxib PK (*47*).

Investigators of rofecoxib PK and metabolism commented on the discordant findings among rats, dogs, and humans. Davies noted that "changes in rofecoxib disposition and pharmacokinetics are evident between species, between races, in elderly patients, and in patients with hepatic or renal disease" (*45*). Halpin (*43,46*) reported that there was little overlap of findings among the three species and concluded that "rofecoxib displayed notable species differences in pharmacokinetic and metabolic behavior." Slaughter, from the same laboratory as Halpin and Baillie, later demonstrated that the complex human metabolic pathway for rofecoxib could be duplicated using human liver subcellular fractions (*48*). Thus, the essentials of rofecoxib metabolism in humans could have been shown more accurately from *in vitro* studies using human tissue than from animal studies.

Celecoxib PK and metabolism have been studied in Sprague-Dawley rats, mice, rabbits, beagles, cynomolgus monkeys, rhesus monkeys, and humans. A single oxidative metabolic pathway was dominant in all species, but significant interspecies metabolic differences were identified among nonhuman species (*49–52*). Sex-related PK and metabolic differences were also found for rats and mice, and may be attributable to sex-specific expression of cytochrome isoenzyme genes (*49,51*). For example, celecoxib plasma t½ was 3.73 hours and 24-hour dose excretion was 80.6% for male rats, compared to 14.0 hours and 32.5% for female rats (*51*).

Celecoxib PK and metabolism in beagles are unique, partly due to the identification of two distinct metabolic phenotypes (*50*). Fast metabolizers displayed plasma celecoxib t½ of 1.72 hours and plasma clearance rate of 18.2 ml/min/kg. Corresponding values for slow metabolizers were 5.18 hours and 7.15 ml/min/kg. Similar to humans regarding rofecoxib metabolism, the demonstration of such gene polymorphisms in dogs means that even individuals within a species may display variable PK and metabolism for the same drug. Susan Paulson, the most prolific investigator of celecoxib PK and metabolism, has stated: "Although the dog is a useful and convenient model for humans, there are differences between the two species that may affect an oral pharmacokinetic profile" (*52*).

In humans, Tmax for celecoxib was 1.42 hours and t½ was 11.5 hours (*53*). Oxidation was the major metabolic pathway, and first pass metabolism was negligible. Terminal urinary excretion was 27.1%, and fecal excretion averaged 57.6% but was quite variable. Human PK and metabolism differed quantitatively from all animal species tested. The influence of a high-fat meal upon celecoxib PK was examined in beagles and humans (*52*). Food delayed celecoxib absorption and prolonged the exposure time for dogs, but had no significant effect for humans.

Valdecoxib PK and metabolism have been evaluated in mice, dogs, and humans. Studies in rats and rabbits have demonstrated that valdecoxib crosses the placenta in both species and enters the cerebrospinal fluid in rats (54). In CD-1 mice, sex-related differences in terminal excretion were identified (*55*). Male mice excreted 38% of the administered drug in the urine and 61.8% in feces; these values were equal for female mice (47.5% and 47.2%, respectively). A larger sex-related difference was shown for distribution into plasma and red blood cells. Tmax for mice was 0.5 hours, and 16 valdecoxib metabolites were identified.

In humans, extensive hepatic metabolism of valdecoxib was demonstrated, using both CYP450 and other metabolic pathways (*54*). Less than 5% of an oral dose was excreted unchanged in urine and feces, and 70–76% was

excreted as urinary metabolites (*54,56*). Nine valdecoxib metabolites were identified in humans. Human Tmax was variably reported to be 1.7–3.0 hours, and t½ was variably reported to be 7–11 hours (*54,56*). In a review of valdecoxib PK and metabolism, the United Kingdom's National Health Service stated that both CYP450 and other metabolic pathways were identified in humans, but that CYP450 activity was absent in dogs and only mildly increased with high multiples of human dosing in rats (*57*). The reviewer commented that valdecoxib "metabolism is complex and varies qualitatively across species." Among the conclusions presented was: "It is therefore not possible to fully elucidate all the potential interactions and their potential clinical impact using pre-clinical studies."

Rofecoxib is metabolized in humans by a combination of CYP450 and other pathways, whereas celecoxib is predominantly metabolized by the CYP2C9 isoenzyme (*58*). Chauret and colleagues performed an extensive evaluation of CYP450 enzyme activities in horses, dogs, cats, and humans (*59*). Seven catalytic activity markers for CYP450-mediated reactions were measured, and Chauret reported that "rather large interspecies differences were observed." Selective CYP450 inhibitors also had widely variable effects among the four species.

Thus, PK and metabolic evaluations of all three COX-2 inhibitors approved in the United States demonstrate important and inconsistent differences related to species, phenotype, and sex. For none of these drugs do animal data predict human PK, metabolism, or toxicity. It appears that pre-clinical animal studies provided only data gathering for the purpose of obtaining FDA approval, and that the actual results of such studies were irrelevant. One may reasonably ask how these results were incorporated into decisions regarding drug approval, and even why the animal studies were required when they contributed no useful information applicable to humans.

IV. Animal and Human Mechanistic Studies of COX-2 Inhibitors

Studies of drug mechanisms and effects for COX-2 inhibitors have been performed in mice, rats, rabbits, and humans. The pharmacological effects of COX-2 inhibition have generally been qualitatively similar, though quantitatively and proportionally different, among experimental animals and humans. COX-2 inhibitors preferentially block the effects of the induced COX-2 enzyme with minimal or no influence upon the constitutive COX-1 enzyme in humans (*28,60,61*). In contrast, nonselective COX inhibitors and most other NSAIDs suppress both enzymes.

In humans, COX-2 inhibitors cause substantial or complete suppression of a protective metabolite, prostacyclin (PGI_2), without effect upon thromboxane A_2 (TXA_2) production. TXA_2 promotes platelet aggregation and adhesion, vasoconstriction, and vascular smooth muscle proliferation, which may be protective in case of injury but is pathogenic in the setting of atherosclerosis. COX-1 mediated TXA_2 suppression mitigates these effects, and is thought to be the mechanism for decreased CV event risk produced by the irreversible COX-1 blocking drug aspirin. PGI_2 promotes vasodilation, while decreasing leukocyte activation and adhesion, inflammatory cellular infiltration, and vascular smooth muscle proliferation. There is evidence that PGI_2 also increases nitric oxide production, enhances atherosclerotic plaque stability, reduces or prevents atherosclerosis progression, and decreases CV event risk (*62*).

Based upon these mechanisms of COX enzyme activity, it has been postulated that selective COX-2 inhibitors may increase CV thrombosis risk by blocking PGI_2 production to leave unopposed TXA_2 activity. This may also explain why CV event risk in COX-2 inhibitor clinical trials has been highest among patients not taking aspirin. However, it has been suggested that even nearly complete PGI_2 suppression does not override the ability of the human vascular endothelium to produce PGI_2 sufficient to inhibit thrombosis. Jaffe and colleagues have demonstrated that even with 90% decreased PGI_2 synthesis in humans, there is sufficient endothelial PGI_2 production to prevent platelet aggregation *in vivo* (*63*).

There is evidence in experimental animals and humans that COX-2 is upregulated in many tissues during inflammation or acute ischemic episodes. Of particular importance regarding the effects of COX-2 expression and suppression, this enzyme has been shown to localize in upregulated fashion in atherosclerotic plaque in mice (*64,65*) and humans (*66–70*), but is not found in normal arteries. COX-2 co-localizes in human athero-

sclerotic plaque with enzymes that produce inflammatory mediators, such as nitric oxide synthase, prostaglandin E synthase, and metalloproteinases (*67,69,71*), suggesting to some investigators that COX-2 worsens atherosclerosis. But the significance of COX-2 association with these known prothrombotic substances is uncertain, and it has been postulated that COX-2 may be protective in atherosclerosis through inhibition of inflammation. These findings have been supplemented by evaluations of the potential beneficial or detrimental influences of upregulated COX-2 for atherosclerosis progression and stability, and thus likely effects of COX-2 inhibitor therapies.

Cheng and colleagues studied carotid artery mechanical injury-induced atherosclerosis in mice deficient in receptors for PGI_2, TXA_2, or both prostaglandins (*72*). Their investigations demonstrated that PGI_2 is an important inhibitor of such atherosclerosis, suggesting that PGI_2 suppression would therefore contribute to atherosclerosis development and progression. Rossoni and colleagues performed studies using perfused rabbit hearts subjected to ischemia and reperfusion (*73*). Pretreatment with aspirin or any of three selective COX-2 inhibitors was associated with a concentration-dependent exacerbation of the ischemic injury. Rossoni concluded that COX-2 has an important protective effect in ischemia, and that COX-2 inhibition worsens ischemic injury. In a model of doxorubicin cardiac toxicity, using male Sprague-Dawley rats, Dowd and colleagues showed that doxorubicin-induced myocardial COX-2 expression increased prostacyclin production and limited cardiac toxicity (*74*).

COX-2 is upregulated in many tissues during inflammation or ischemia, including the stomach. Studies in mice and rats showed that COX-2 expression in gastric ulcers contributed to healing, and that healing was delayed by COX-2 inhibition (*75*,76). This finding is contrary to expectation, since COX-2 inhibitors are postulated to decrease the risk for gastric ulcers by sparing gastroprotective COX-1.

There are also studies suggesting a detrimental effect for COX-2, and thus a beneficial or protective effect for COX-2 inhibitors. Saito and colleagues reported that COX-2 induction in ischemic rat myocardium increases pro-inflammatory prostaglandins and contributes to cardiac dysfunction (*77*). COX-2 has also been shown to mediate the synthesis of angiogenic factors which contribute to expansion of atherosclerotic plaques in humans (*66*).

Using a model for lipopolysaccharide (LPS)-induced endotoxemia in male Sprague-Dawley rats, Hocherl and colleagues showed that COX-2 derived prostaglandins produced the adverse CV effects of endotoxemia (*78*). In another study of LPS-induced endotoxemia, using female CD-1 mice, LPS produced a time-sensitive increase in harmful PGE_2 (*79*). Maclouf and colleagues demonstrated COX-2 mediated synthesis of mitogenic prostaglandins in activated human monocytes, producing vascular cell proliferation, vasoconstriction, and atherosclerosis (*80*).

Results from the veterinary literature are also informative regarding species differences for COX2 inhibitors and other NSAIDs (*81–84*). Carprofen (Rimadyl) is a relatively selective COX-2 inhibitor in dogs, but not when tested against human synovial cells. Etodolac (Lodine) and meloxicam (Mobic) are predominantly COX-2 inhibitors in humans, but have shown variable COX selectivity in dog studies (ranging from marginally COX-1 selective to strongly COX-2 selective). Piroxicam (Feldene) and tolfenamic acid are relatively COX-1 selective in humans and COX-2 selective in dogs. The human drugs rofecoxib and celecoxib are not useful for dogs, as their metabolism is not predictable in that species (*85*).

Deracoxib (Deramaxx) was developed to provide greater COX-2 selectivity than carprofen in the treatment of arthritis and other pain in veterinary medicine. Approved by the FDA for use in dogs in 2002, this drug has a predictable duration of action and dose response in dogs, and a reasonable safety profile in postmarketing surveillance. As safe and effective as deracoxib is for dogs, it is lethal for cats, which are unable to metabolize NSAIDs effectively due to diminished glucuronyl transferase activity. Novartis Animal Health U.S., Inc., received an FDA warning letter dated November 29, 2004, because the company failed to report the deaths of 14 cats in an unapproved clinical trial (*86*). Even when humans are not included, animal studies do not translate to other species.

Thus, animal and human studies of COX-2 mechanisms and effects are inconsistent and unpredictable, just as

discussed above for PK and metabolic studies. The reasons for this are fundamental and immutable, as stated by Brian Mandell in a review of COX-2 selective drugs for Cleveland Clinic: "The roles of COX-1 and COX-2 vary among animal species" (*87*). The comments of Matthew Weir in his review of selective COX-2 inhibition and cardiovascular effects are instructive regarding the utility of these mechanistic studies, particularly since he was writing on behalf of Merck and all three of his co-authors were Merck Research Laboratories employees:

> The relevance of these animal models in predicting effects in humans is uncertain, since COX-2 inhibition does not produce a 100% obliteration of prostacyclin nor does it affect receptor function…
>
> Although animal data have not been consistent…, these findings have raised the possibility that COX-2 inhibitors could actually decrease the incidence of acute thrombotic events…
>
> The effects of COX-2 inhibition have been studied in several experimental models including myocardial ischemic preconditioning, chemotherapeutic-associated cardiomyopathy, and surgically induced myocardial infarction, with conflicting results…
>
> The possibility of a neutral, harmful, or even beneficial effect have all been raised (*88*).

V. Animal Studies of COX-2 Inhibition

Outcome-based studies of COX-2 inhibition have been performed in mice, rats, rabbits, dogs, and humans. (Important human clinical trials were discussed earlier in this paper.) Studies in LDL receptor-deficient (LDLR-D) mice have provided variable results. In a study of male LDLR-D mice fed a high-fat rodent chow diet for six weeks, Burleigh and colleagues reported that both the nonselective NSAID indomethacin and the selective COX-2 inhibitor rofecoxib produced smaller aortic atherosclerotic areas than controls (*89*). In a longer study (18 weeks) with fewer LDLR-D mice (male and female) on a similar diet, Pratico and colleagues demonstrated 55.4% decreased atherosclerotic lesion area with indomethacin compared to placebo, but a lesser (30%) and statistically insignificant decrease with the selective COX-2 inhibitor nimesulide (*64*).

Using male LDLR-D mice, Linton and colleagues compared aortic atherosclerosis lesion areas for rofecoxib- or indomethacin-treated mice compared to control mice (*90*). Both drugs resulted in smaller atherosclerotic lesion areas. In a related study, LDLR-D mice null for macrophage COX-2 had smaller lesions than LDLR-D mice wildtype for macrophage COX-2 (*90*). Linton concluded that COX-2 has an atherogenic effect which is blocked by rofecoxib and indomethacin, that genetic evidence suggests an atherogenic role for macrophage COX-2 expression, and that COX-2 inhibition may be therapeutic for atherosclerosis.

Three studies of COX-2 inhibition in ApoE knockout mice also have had mixed results. Heeschen and colleagues studied mice treated with nicotine ± rofecoxib or rofecoxib alone, compared to control mice (*91*). Nicotine-treated mice had doubling of atherosclerotic lesion area compared to controls, and greater lesion vascularity. Both pathological effects of nicotine were abolished by rofecoxib. A short-term study of oral treatment with the selective COX-2 inhibitor MF-tricyclic demonstrated increased atherosclerotic lesion area with COX-2 inhibitor treatment (*92*). A longer study demonstrated smaller lesions with rofecoxib, NS-398, and indomethacin, compared to control mice (*93*).

Thus, two of three studies in ApoE knockout mice and three of four studies in LDLR-D mice demonstrated an apparent protective effect for COX-2 inhibition in atherosclerosis; the fourth LDLR-D study showed a similar but statistically insignificant trend. These results are contrary to the results of the human clinical trials discussed above. Corruzi, in his review of animal studies involving NSAIDs, stated: "Results obtained in these studies, however, must be extrapolated with caution to those observed with pharmacological therapy in patients, since gene knockout animals may undergo compensatory mechanisms" (*94*). Burleigh commented upon the inadequacy of the mouse model for human atherosclerosis: "Although the mouse is a widely used model for the investigation of atherosclerosis, the absence of plaque rupture and coronary thrombosis leading to myocardial infarction are clear limitations of the mouse as a model for human coronary artery disease" (*89*).

Two studies of the effects of COX-2 inhibition in doxorubicin cardiac toxicity showed conflicting results. In male Sprague-Dawley rats, the selective COX-2 inhibitor SC236 blocked synthesis of the protective prostacyclin, resulting in increased cardiac injury (*74*). In a mouse model of doxorubicin-mediated heart failure, COX-2 inhibition resulted in improved cardiac function (*95*).

Using a coronary artery ligation model of myocardial infarction in Lewis rats, Saito and colleagues demonstrated improved cardiac function four weeks after infarction in rats treated with the selective COX-2 inhibitor DFU, compared to placebo (*77*). Saito concluded that COX-2 contributes to postinfarct cardiac dysfunction, and that COX-2 inhibition may be a therapeutic measure for myocardial infarction. In a coronary artery ligation model using female Wistar rats, Scheuren and colleagues demonstrated decreased infarct-related inflammation and fibroblast proliferation in rats treated with oral rofecoxib, compared to controls (*96*). In a coronary artery ligation model using mice, LaPointe and colleagues identified COX-2 expression in infarcted hearts but not in control hearts (*97*). Mice treated after infarction with rofecoxib or NS-398 had less cardiac damage than control hearts. LaPointe concluded that COX-2 expression in myocardial infarction may contribute to pathological left ventricular remodeling, and that COX-2 inhibition may mitigate these effects.

Using male Sprague-Dawley rats, Yang and colleagues evaluated the effects of celecoxib following mechanical denudation injury to the carotid artery (*98*). Celecoxib decreased vascular smooth muscle proliferation and neointimal hyperplasia after denudation, and Yang suggested a potential role for celecoxib to prevent restenosis after coronary artery angioplasty. No studies have been conducted in humans to evaluate the effects of COX-2 inhibition in patients during or after myocardial infarction, or after percutaneous revascularization. Furthermore, it is unlikely that such studies will occur, since, contrary to the results of these animal studies, all three FDA-approved COX-2 inhibitors have been shown in human clinical trials to increase CV events in patients with documented atherosclerosis, increased risk for atherosclerosis, or recent coronary bypass surgery.

Shinmura and colleagues evaluated the influence of COX-2 in conscious rabbits modeling ischemic preconditioning (*99*). Intermittent coronary occlusion and reperfusion produced marked upregulation of myocardial COX-2 mRNA, COX-2 protein, and other COX metabolites. This ischemic preconditioning diminished the extent of myocardial stunning and infarction produced by subsequent sustained coronary occlusion.

Shinmura concluded that this protective effect is mediated by prostaglandins produced by upregulated COX-2. The effects of selective COX-2 inhibitors celecoxib and NS-398 upon prostaglandin synthesis and infarction were also evaluated. When administered 24 hours after preconditioning, both drugs abolished the increases in COX metabolites and eliminated the protective effect of ischemic preconditioning.

Guo and colleagues performed a similar evaluation in B6129F$_2$/J mice (*100*). Ischemic preconditioning with six cycles of coronary occlusion and reperfusion resulted in decreased infarct size following sustained occlusion. Guo concluded that upregulated COX-2 mediates the protective effect of late phase ischemic preconditioning in this mouse model. NS-398 had no effect upon infarct size produced by sustained coronary occlusion, compared to control mice. However, when administered after ischemic preconditioning (30 minutes before sustained coronary occlusion), NS-398 abolished the protective effect of ischemic preconditioning.

Hennan and colleagues evaluated the effects of COX-1 inhibition with aspirin and COX-2 inhibition with celecoxib, using a dog model of myocardial infarction produced by coronary artery electrolytic injury (*101*). Aspirin prolonged the time to occlusion after electrolytic injury, but this prolongation was abolished by adding oral celecoxib. Hennan suggested that this result was due to celecoxib elimination of COX-2 mediated protective prostacyclin synthesis. No human studies have been performed to evaluate COX-2 inhibition during acute coronary syndromes, nor are any likely to occur for reasons stated above.

In an interview with the *New York Times* News Service on October 4, 2004, Pfizer vice president Mitch Gandelman noted that the results of animal studies using Celebrex varied, and that such studies did not always reflect what happens with people (*102*).

VI. Why Animal Models Were Misleading in COX-2 Inhibitor Development

It is apparent that animal research conducted for COX-2 inhibitor drug development has not been translatable to the human experience. The fundamental reasons for this relate to evolution and biology. The imperative of biological diversity, produced by natural selection, mutation, and adaptation over evolutionary time, has resulted in divergence of species. Even among humans there is some biological diversity, despite our relatively short evolutionary history. This divergence has produced important biological differences among species, including anatomy, physiology, metabolism, and genetics.

Some of these important species biological differences are evident at the gross anatomical level. For example, although dogs are commonly used to evaluate coronary artery disease and heart attacks (including responses to drugs), their coronary anatomy and pattern of myocardial perfusion are quite different from humans' (*103*). Because they do not develop atherosclerosis naturally, heart attacks are simulated by coronary artery ligation, bead occlusion, or electrolytic thrombosis. In contrast, heart attacks in humans typically occur as the result of decades of atherosclerosis progression, terminating in plaque rupture often associated with inflammation. The resulting infarctions are not even comparable to those seen with human heart attacks, because dogs have different coagulation parameters and extensive coronary collateral circulation.

Similarly, the typical mouse and rat models for stroke research are a contrivance developed for convenience rather than scientific validity. Mice and rats also do not develop atherosclerosis naturally, and vascular damage is often produced in these models by mechanical disruption of the carotid arteries (*72,98*). Strokes are produced by ligation or thrombosis. Mouse and rat cerebral vascular anatomy, collateral circulation, and physiological responses to stroke are so different from humans' that even researchers in the field acknowledge the inadequacy of the models. Ness stated that "The repeated failures of laboratory proven stroke therapies in humans can be due only to the inapplicability of animal models to human cerebral vascular disease" (*104*). Wiebers observed:

> Ultimately, the answers to many of our questions regarding the underlying pathophysiology and treatment of stroke do not lie with continued attempts to model the human situation more perfectly in animals, but rather with the development of techniques to enable the study of more basic metabolism, pathophysiology and anatomical imaging detail in living humans (*105*).

These factors help explain why so many drugs are effective for treatment of strokes in animals, yet ineffective for humans. Species differences are also evident at the level of organ structure and function. Rats do not have gall bladders, which may influence drug metabolism due to the inability to concentrate bile (*106*). The rat's unique enterohepatic circulation was discussed regarding rofecoxib metabolism, as were the differences in rofecoxib gastrointestinal absorption among rats (nearly 100%), dogs (36%), and humans (variable with dose). Rats and rabbits have relatively permeable placentas, and many drugs that cross the placenta in these species are safe during human pregnancy. However, the opposite can also be true, as evidenced by failure of teratogenicity testing to explain the thalidomide disaster even after the fact (*107*).

Blood-brain barrier permeability also differs substantially among species, making evaluation of central nervous system (CNS) drug distribution and toxicity in animals not useful for humans. Mice and rats restrict drug transport into the CNS much more than do chickens, hamsters, rabbits, cats, monkeys, and chimpanzees (*108*). Such differences may help explain unanticipated neurotoxicities in humans from zimeldine, clioquinol, pimozide, maprotiline, buproprion, benzodiazepines, and many other drugs. Opioids produce widely variable CNS depressant or stimulant effects in different animal species, confirming species-dependent responses even when the blood-brain barrier is crossed (*109*).

Species differences in liver detoxification capacity may explain why many drugs are safe in animals, despite the fact that liver toxicity is the major reason for drug relabeling and withdrawal in humans (*110*). In the great majority of cases, resulting plasma drug t½ is significantly shorter for experimental animals than for humans. As an example of variable toxicity related to hepatic metabolism, diazepam can cause fatal liver failure in cats at low doses, but very large doses may be required for

seizure control in dogs (often 2–3 mg/kg body weight), due to efficient hepatic detoxification (*111*). An equivalent dose would be rapidly lethal for humans.

Similarly, species differences are evident at the metabolic, cellular, subcellular, and gene levels. Comparative animal and human metabolic studies, as discussed earlier, routinely demonstrate variable metabolic pathways among species. Exemplary is the interspecies variability of aspirin metabolism, which has plasma t½ of 15–20 minutes for humans, 30 minutes for cows, 1 hour for horses, 4.5–8.5 hours for dogs, and 27–45 hours for cats (*112*). Metabolic products are also different in number and structure for each species, and may be toxic or lethal for some species while safe for others. As John Caldwell noted in his review of animal drug toxicity testing:

> The occurrence of major quantitative and qualitative differences between animal species in the metabolism of xenobiotics is well documented. Interspecies differences in metabolism represent a major complication in toxicity testing, being responsible for important differences in both the nature and magnitude of toxic responses…In particular, these differences represent probably the single greatest complicating factor in the use of animal toxicity data as an indication of potential human hazard (*113*).

Important biological characteristics, such as those that determine disease expression, drug efficacy, and toxicities, do not just vary by species. Rather, there are major differences within species as well, such as fundamental sex differences in mice and rats regarding atherosclerosis expression, drug metabolism, toxicities, and efficacy (*114–116*). Differences in drug toxicities have also been documented within the human species on the basis of age, race, ethnicity, and sex (*117*).

Genetic differences in the structure and function of genes can alter protein synthesis and regulation in ways that invalidate intra- and interspecies correlations. Variations in gene sequences within specific regions of the genome result in differential gene expression and protein synthesis, producing functional variants called polymorphisms. For example, there is great intra- and interspecies genetic diversity involving the hepatic CYP450 enzymatic pathway, the major drug detoxification enzymatic system for humans. There are more than 1,500 CYP450 genes in nonhuman animals, more than 500 in vertebrates, and 63 human genes coding for CYP450 enzymes (*118,119*). Gene polymorphisms have been identified in humans for poor, intermediate, efficient, and ultrarapid metabolizers of drugs using the human CYP2D6 isoenzyme, with consequences ranging from no drug effect to serious drug toxicity (*120*). There are hundreds of human CYP450 isoenzyme polymorphisms (more than 80 for CYP2D6 alone), that affect correlation of drug metabolism, efficacy, and toxicity among and within species (*120*).

Another example of human genetic diversity is the extent of polymorphism in the gene regions related to lipid metabolism. Chasman and colleagues identified 148 single nucleotide polymorphisms (SNPs) within 10 such gene regions, including 33 SNPs in the HMG-CoA reductase gene alone (*121*). The researchers reported that polymorphisms in the HMG-CoA reductase gene correspond to variable responses of total and LDL cholesterol levels in patients receiving pravastatin. Variable clinical responses to pravastatin have also been described in relation to polymorphisms in the cholesterol ester transport protein gene (*122*).

Polymorphisms in the serotonin neurotransmitter receptor gene have been linked to mephenytoin responses (*123*). Many other specific links have been identified between gene polymorphisms and drug responses. It is estimated that more than 90% of human genes display polymorphisms—a conclusion that has contributed to the development of new scientific disciplines such as toxicogenomics, proteomics, pharmacogenetics, and pharmacogenomics (discussed in the next section).

Many research animal models are genetic inventions intended to mimic susceptibility or disease states in humans. There is almost an unlimited selection of such gene knockouts or mutated animals, particularly of mice and rats. Several of these artificial species were discussed regarding COX-2 related animal tests (*43,49,55, 64,72,77,89,91,95*). Because these animals are post hoc attempts to create the circumstances of human disease, they do not reflect the true processes by which humans contract such diseases.

For example, the LDLR-D mouse strain was developed to promote hypercholesterolemia and atherosclerosis

for convenient laboratory studies, but the factors that cause these conditions in humans were not employed. The created pathology does not mimic human atherosclerosis, as evidenced by previously noted pathological differences: plaque in the LDLR-D mouse is limited to the aorta, is focal rather than diffuse, and does not rupture or thrombose (*89*).

Furthermore, artificial and unnatural methods are used in experimental animals to produce pathologies and events for which preventive and therapeutic interventions including drug therapies may be tested. Mice and rats in COX-2 studies received paw pad or pleural injection of carrageenan, or aural arachidonic acid injection, to produce pain and inflammation (*124–126*). Other studies have utilized intraperitoneal injections of lipopolysaccharide (*78,79*) to produce endotoxemia, and gastric disruption with acidified ethanol, carbachol, acid instillation, and ischemia-reperfusion to induce gastric ulcers (*94*). Such methods are required to produce pathology in the COX-2 studies and most other animal studies of human pathology because exposure to human risk factors or pathogens does not work. These artificial diseases do not reflect human pathology or responses, but are in fact different diseases entirely.

In this regard, Stephen Kaufman noted that "Because animal experimentation focuses on artificially created pathology, involves confounding variables, and is undermined by species differences in anatomy and physiology, it is an inherently unsound way to investigate human disease processes" (*127*). Op Flint of Bristol-Meyers Squibb stated the crux of the matter by noting that "it is impossible to establish the reliability of animal data until humans are exposed" (*128*). And once human data are available, animal data are even less relevant or justifiable.

Note too that among the most common or troublesome human side effects from approved drugs are headache, nausea, dizziness, fatigue, weakness, myalgias, arthralgias, memory deficits, and depression. These and many other side effects cannot be obtained from research animals, and thus cannot be predicted for humans.

Such basics of laboratory animal studies as manual handling, blood drawing, intravascular or intracavitary injections, orogastric gavage, vascular or other instrumentations, and anesthesia produce profound and lingering physiological alterations (*105,129*). Even such routine measures as entering an animal's room, moving its cage, using different types of bedding, lighting, noise, water availability, and dietary changes may alter animal behavior and physiology. Typical alterations include behavioral changes (anxiety, fear, hyperactivity), increases in biochemical stress markers (corticosterone, epinephrine and norepinephrine, glucose, thyroid hormones, growth hormone, prolactin), and increases in physiological stress markers (blood pressure and heart rate) (*129*). The introduction of physical and mental stress, with the attendant physiological disruption, is inseparable from manipulation of the animals for evaluation. Such changes likely compromise or invalidate data obtained from the animals.

It is common knowledge that animal studies often do not produce postulated results–or even interpretable results. With few exceptions, such studies are not made available for review or analysis but are discarded. Animal studies are also susceptible to manipulation, such as alteration of protocols, exclusion of outliers, elimination of inconsistent data, or even fabrication of data to fit study hypotheses. The same degree of oversight required for human clinical trials does not accompany animal studies. Research and publication misconduct has been well documented for animal and clinical research, even at highly regarded research institutions and in the most respected medical journals (*130–144*).

A particularly serious weakness in using animal research to evaluate drugs for human use is the role of sponsoring companies in the conduct and reporting of animal studies. Animal test results, like the human clinical trial data discussed previously, are susceptible to being suppressed if unfavorable, massaged if workable, and oversold if favorable. Industry-sponsored clinical trials have been shown to be two to four times more likely to produce results favorable to the industry sponsor than are independent trials (*145–148*).

In light of these considerations, scientists are increasingly questioning whether animal models can produce reasonable, predictable, or reproducible approximations of drug metabolism, efficacy, or toxicity for humans.

VII. Replacing Animals in Pharmaceutical Research and Drug Development

Human clinical pharmacology, typically phase I and phase II clinical drug trials, are the first steps in the current drug development process that actually address human responses. Phase I trials are small studies (usually 20-100 healthy volunteers) investigating drug absorption, distribution, metabolism, excretion, and toxicity (ADMET), using small and gradually increasing doses of the investigational drug. These trials frequently identify drug ill effects not suspected from animal studies, and about 40% of candidate drugs are eliminated during phase I trials. Phase II trials are larger studies (usually several hundred patients) designed to obtain preliminary evidence about the efficacy of a drug for specific medical conditions. These studies also provide a larger look at short-term side effects and toxicities. Both phase I and phase II trials commonly refute animal data regarding ADMET, side effects, and efficacy, and most candidate drugs do not progress past human pharmacological trials.

Microdosing technology is a relatively recent improvement upon the traditional methods of human clinical pharmacology. This technology permits the use of radiolabeled trace doses (1-100 mcg) of candidate drugs to evaluate absorption, distribution, metabolism, and excretion in humans. These doses are less than 1 percent of that required to produce a pharmacological effect, and thus there is virtually no risk for adverse effects. The radiation exposure is less than that obtained during a four-hour airplane flight. Positron emission tomography is used to acquire real-time data regarding drug disposition, and accelerator mass spectrometry is used to analyze parent drug and metabolite concentrations in blood, urine, and feces at specific intervals after dosing. Accurate analyses of drug distribution volume, Tmax, Cmax, time-concentration curves, and plasma t½ are thereby acquired in humans.

Microdosing technology is commercially available and has many procedural, economic, and validity advantages for drug manufacturers and regulatory agencies (*149,150*). Microdosing technology was endorsed by the European Agency for the Evaluation of Medicinal Products in January 2003 (*151*), and has already been used to identify drug candidates for human phase I trials (*152,153*). Microdosing was developed to minimize preclinical drug evaluation and early clinical drug attrition by using single-dose "phase 0" human studies. This method should replace pharmacological animal tests, which have little or no relationship to human pharmacology.

Aside from microdosing technology, many companies are pursuing other approaches to human ADMET. Among the tools being developed, tested, and documented are computer models and simulation programs for human drug PK and metabolism, performance software for ADMET procedures, methods to use human tissues for *in vitro* ADMET testing, and refinement of testing methods. One of the dozens of companies working in this field is Pharmagene, which does no animal-based testing because, as it states, "Using human tissue allows you to investigate the role of targets of interest or the actions of test compounds in the target species, man. Using human tissue allows you to select the best targets and the right compounds at the earliest stage, thus reducing the chances of failure in the clinic" (*154*). Pharmagene and many other companies have huge banks of normal and diseased human tissues and human cell lines. These are available for ADMET and drug efficacy testing, and many partnerships with pharmaceutical companies are already in place.

By combining structural chemistry, mathematical models, and computational methods, scientists are able to produce accurate computer-based (*in silico*) human ADMET data. Using known biochemical and physical consequences of molecular structure, drug and chemical testing may be performed by generating qualitative and quantitative structure-activity relationships (SAR). This technology allows human ADMET prediction which may equal or exceed the accuracy of *in vitro* methods. Computer predictive models are helping to create rules-based criteria used to describe SAR, to predict toxicities and carcinogenicity, and to contribute to drug selection and design (*155*). In situations when *in vitro* and *in silico* methods are equally accurate, the latter technique may be able to shorten drug and chemical screening time from days or weeks to just minutes.

Human in vitro testing is another excellent tool to assess drug toxicity and efficacy. Advances in cell and tissue preservation technology have allowed scientists to construct, maintain, and analyze complex human tissue

cultures and cell layers. Superiority of human tissue *in vitro* methods to animal studies was demonstrated years ago (*156–158*), and the superiority gap has widened with improved systems and greater tissue availability. Virtually all types of human tissue are now being studied using *in vitro* techniques to elucidate disease mechanisms, drug targets, efficacy, and toxicity.

The Multicentre Evaluation of *In Vitro* Cytotoxicity was established as an international program to develop optimal accuracy for human drug toxicity testing, using 50 reference chemicals and a battery of 61 human cell line cytotoxicity assays (*159*). During the study period 1989-1996, twenty-nine laboratories performed a complete series of toxicity tests under the auspices of this program. Results were compared to the standard LD50 animal toxicity tests, using human acute toxicity data regarding toxic and lethal blood and tissue concentrations as comparative standards. A battery of three human cell line assays was superior to animal LD50 testing for prediction of human acute toxicity; the predictive value of the assays was increased by incorporation of human toxicokinetic data.

The National Cancer Institute's Developmental Therapeutics Program completed its *In Vitro* Cell Line Screening Project from 1985 to 1990; the program became fully operational in April 1990. This project was designed to provide high-volume drug screening for potential anticancer agents, and arose from "dissatisfaction with the performance of prior *in vivo* [animal] primary screens" (*160*). The screen uses 59 human tumor cell lines to evaluate anticancer effects of candidate drugs, and has replaced animal testing for this purpose at the National Cancer Institute. The method is sufficiently sophisticated to produce pattern recognition algorithms from responses of the cell lines to specific drugs. Using these algorithms, drug mechanisms of action may be evaluated, cell line molecular targets characterized, and drugs selected for their abilities to interact with specific molecular targets.

Genetically engineered human monoclonal antibodies are being studied and used for cancers, immunological disorders, psoriasis, and other disorders. A novel approach to human HIV therapy was suggested by an *in vitro* study demonstrating that the opioid receptor antagonist naltrexone potentiates the antiviral activity of AZT and indinavir (*161*). Potential new dietary treatments for human *Helicobacter pylori* infections resulted from an *in vitro* study demonstrating that sulforaphane kills the bacterium and inhibits gastric tumor formation (*162*). Recombinant DNA research using *in vitro* methods has had dramatic benefits, contributing to the development of such products as human insulin, hepatitis B vaccine, reteplase (recombinant tissue plasminogen activator), erythropoietin, human growth hormone, clotting factors, and α-galactosidase A (the missing enzyme in Fabry's disease) (*163–165*). The applications of human *in vitro* technology are almost limitless, and provide insights and treatments directly applicable to humans rather than to animal models.

The use of *human stem cells* is an exciting and potentially highly productive methodology, which has applications for drug testing and development, toxicity testing, targeted disease treatments, and gene therapies. Stem cells are precursor cells capable of differentiating into any of the approximately 220 types of human specialized cells. *Adult stem cells* are multipotent cells obtained from organs such as bone marrow, brain, and liver that have the ability to differentiate into the cell types specific to their organs. Multipotent stem cells may also be obtained from placentas and umbilical cord blood, and may have greater differentiation capability than adult stem cells. *Embryonic stem cells* may be harvested from aborted embryos or unused embryos from fertility clinics, and have totipotent differentiation capability. Stem cells also may be obtained by *cloning* cells from the DNA of humans who would receive the cells (therapeutic cloning or somatic cell nuclear transfer) (*166,167*). Therapeutic cloning and embryonic stem cell research involving donated embryos from *in vitro* fertilization were approved for medical research purposes in Britain in 2001 (*168*). Scientists generally agree that embryonic stem cells have the greatest potential to produce innovative and targeted human therapies than other stem cells, but the use of embryonic stem cells is controversial due to the manner in which they are obtained.

Stem cells may be used to test the toxicities and efficacies of drugs, chemicals, or other substances, or may be used to grow cell populations or tissues for toxicity testing or therapeutic purposes. Much of the enthusiasm for stem cell research relates to the potential for patient-specific treatment of disorders such as Parkinson disease, Alzheimer disease, ALS, solid and hematological cancers, diabetes, multiple sclerosis, juvenile rheumatoid arthritis, systemic lupus erythematosus, scleroder-

ma, spinal cord injury, stroke, and heart attack. Human stem cells may be used to grow hepatic, CNS, or other cells to test drug toxicities specific to humans—bypassing unreliable animal tests. Human stem cell genes can be deleted or replaced (homologous recombination), allowing evaluation of gene defects and targeted gene therapies (*169*).

Perhaps the ultimate goal in evaluating drug ADMET and efficacy in humans is to find the means to target drug therapies based on individual genetic profiles predictive of response and toxicity. Even though human intraspecies genomic homology is thought to be 99.9%, there is still potential for genetic differences to produce clinically important safety and efficacy concerns. The Human Genome Project has identified approximately 30,000 human genes, consisting of more than 3 billion base pairs and coding for more than 100,000 proteins. Even with only 0.1% gene variability, there may be as many as 3 million differential gene polymorphisms between two humans, and even monozygotic twins have displayed different drug toxicities. A subprogram of the Human Genome Project—the Human Genome Diversity Project—investigates the nature and consequences of diversity in the human genome (*119*).

The sciences of toxicogenomics, proteomics, pharmacogenetics, and pharmacogenomics directly address the issues of human genetic diversity and the consequences regarding drug toxicity and efficacy. *Toxicogenomics* is the study of gene functions related to toxicology. *Proteomics* is the study of the structure, function, and interactions of proteins. *Pharmacogenetics* is the study of inherited differences in drug metabolism and responses. *Pharmacogenomics* is the study of genes that influence drug responses; in particular, how genetic differences may predict drug efficacy and toxicity. The latter two terms are often used interchangeably to denote the broad study of genetic variability as it relates to human pharmacology and drug therapy (*170*).

At the clinical level, pharmagenomics has the potential to personalize drug delivery to maximize therapeutic responses and minimize toxicities. For example, identifying specific enzyme polymorphisms by detection of corresponding SNPs can predict which categories of drugs (or which specific drugs) may display metabolic characteristics predictive of toxicities or of efficacy. Variable expression of CYP450 isoenzymes, HMG CoA reductase gene segments, and many other determinants of clinical responses to drug therapies will permit personalized pharmacology with high predictive value. Had such capabilities been available, patients at risk for lethal toxicities from cerivastatin, troglitazone, cisapride, and many other drugs might have been excluded from taking them.

At the drug development level, pharmacogenomics will expedite and streamline clinical trials by segregating patients with favorable or unfavorable genetic profiles for the candidate drugs. Clinical drug trials would exclude subjects with genetic profiles predictive of toxicity or inefficacy, eventually allowing more accurate results with smaller trials of shorter duration. When combined with appropriate *in vitro* and/or *in silico* drug testing and with human phase 0 microdosing studies, the entire process of drug identification, screening, and clinical testing may be shortened. Potential advantages related to broad application of pharmacogenomics include better drug design through identification of genome targets, faster and more efficient drug development, decreased candidate drug attrition, decreased size and duration of clinical trials, better determination of drug dosing, improved predictive accuracy for therapeutic and toxic effects, decreased adverse drug reactions, increased number of drugs available to selected patients, and rescue of previously denied or withdrawn drugs for applications in appropriate patients. Many of these advantages translate into tremendous cost savings related to drug development, testing, and monitoring; these in turn may result in lower drug costs to patients.

A limiting factor in the broader application of pharmacogenomics is the time and expense associated with the gene sequencing required to identify SNPs. This problem is being addressed successfully by the development of DNA micoarrays (chips), which permit screening for tens of thousands of SNPs within a few hours. Maturation of DNA microarray technology is expected to lead to a time when patients will be screened rapidly in their doctors' offices to determine drug efficacy and toxicity before receiving a drug prescription. This approach is light years away from guessing about such responses on the basis of unreliable animal tests.

The FDA has acknowledged the tremendous potential for pharmacogenomics to change how drugs are developed, tested, and prescribed. In the original FDA docu-

ment promoting pharmacogenomics and urging the pharmaceutical industry to incorporate such methods for drug development, Lesko commented: "The process of drug discovery may be transformed by this knowledge" (*171*). There followed in November 2003 an FDA guidance document for pharmaceutical industry submission of pharmacogenomics data (*172*). In December 2004, the FDA emphasized the importance of this technology by establishing a pharmacogenomics subcommittee in the Office of New Drugs (*173*).

Later that month, the FDA approved the first DNA microarray test for clinical use, the AmbliChip Cytochrome P450 Genotyping Test from Roche Molecular Systems, Inc. (*174*). The microarray analyzes cytochrome P450 isenzymes CYP2D6 and CYP2C19, which metabolize about 25% of drugs used in humans (*120*), and was approved for use with the Affymetrix GeneChip Microarray Instrumentation System, manufactured by Affymetrix, Inc. A review of societal and technical aspects of pharmacogenomics in drug development also was published in 2004, describing specific applications of the technology and the legal, industry, and regulatory changes required to make this approach successful (*175*). Collaboration, cooperation, and commitment of all parties to the development of pharmacogenomics would accelerate entry into a fundamentally different and superior process for drug identification, development, and delivery.

Human clinical pharmacology, microdosing technology, *in vitro* and *in silico* approaches to human ADMET and candidate drug assessments, human stem cell technology, and pharmacogenomics will provide data far superior to current animal testing and typically limited clinical trials. Even those who adhere to animal research dogma must now admit that its continued use is an anachronism in view of available replacements. If we are to realize the potential of available technologies, and maximize therapeutic drug safety and access, we must stop animal testing and shift resources toward the development and application of replacement methods. Only in this way can health disasters such as Vioxx and its many toxic predecessors be eliminated.

VIII. Recommendations to Improve Pharmaceutical Development and Delivery in the United States

Based upon the foregoing information, the following recommendations are made:

1. The FDA should delete animal testing requirements from the drug development and approval process, because such testing is misleading and harmful for humans.

2. The pharmaceutical industry should be liable when harm to humans results from reliance on animal safety studies, because such studies have no relevance for human risks.

3. Preapproval pharmacological study protocols should be available in a database for public access, and the FDA should require that original data from all registered protocols be submitted for expert review with all IND applications.

4. Specific guidelines should be developed for the inclusion of appropriate *in vitro*, *in silico*, microdosing, stem cell, and pharmacogenomics data with all new drug applications.

5. Federal research funding programs should shift research funding from animal-based drug research to superior replacement methods, in order to promote development of those methods. Minimum programmed reductions in animal research funding should be included; for example, a 25% reduction in years one and two, a 50% reduction in year three, a 75% reduction in year four, and a 90% reduction in year five.

6. Larger and longer phase II and phase III human clinical trials should be required, until the pharmaceutical industry develops the superior technologies that will permit more accurate results with smaller and shorter clinical trials.

7. Mandatory regulated phase IV human clinical studies (postmarketing surveillance) should be instituted, and these should be regulated by an agency separate from the FDA to prevent conflict of inter-

est. Continued regulatory drug approval should be contingent upon favorable review of such studies.

8. Strict conflict of interest guidelines should be applied to relationships among the FDA, its sub-agencies, and the pharmaceutical and research industries. Strong whistleblower protection should be part of these guidelines.

9. Strong sanctions should be prescribed for unethical or dishonest actions by any parties responsible for the design, development, performance, and reporting of information for the purpose of obtaining drug or device regulatory approvals.

These or similar measures will be needed in order to fulfill expectations and obligations to protect the public, and to regulate the commercial pharmaceutical industry in a manner conducive to the public health.

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