

# COX-2 inhibitor toxicity *warning shot over pharma's bow*

Human beings are complex 'gene machines' that rely on the intricate interplay of ~25,000 genes to impart biological function. Genomic function is affected by genetic variability and environmental factors, giving rise to considerable functional heterogeneity in the human population. Drug responsiveness and side-effects can vary from person to person, as seen in the recent observation of COX-2 inhibitor toxicity in a subset of prescribed users. Currently available microarray assays for genotyping and genome-wide expression analysis offer prospects for reducing the incidence of COX-2 inhibitor toxicity by providing diagnostic information regarding susceptibility to cardiovascular complications. The same technologies may be used in an attempt to select patients most likely to benefit from novel therapies (for example, selecting people more likely to be at risk of gastrointestinal complications from NSAID use, the driver for development of COX-2 selective inhibitors in the first place). The extent to which the pharmaceutical industry embraces these recent events as an opportunity to improve drug safety hinges on whether COX-2 inhibitor toxicity is seen as the 'warning shot' that it is.

**H**uman beings can be viewed as 'gene machines' that rely on the genes encoded by the genome to mediate and direct biological function. This biological model is analogous to the manner in which mechanical devices (eg computers, robots, wristwatches) derive proper function from the concerted action of semiconductors, resistors, gears, springs and other mechanical parts (Figure 1). Mechanical and electronic parts in the machines that human ingenuity has invented interact with each other and the environment in the performance of their roles. While built to with-

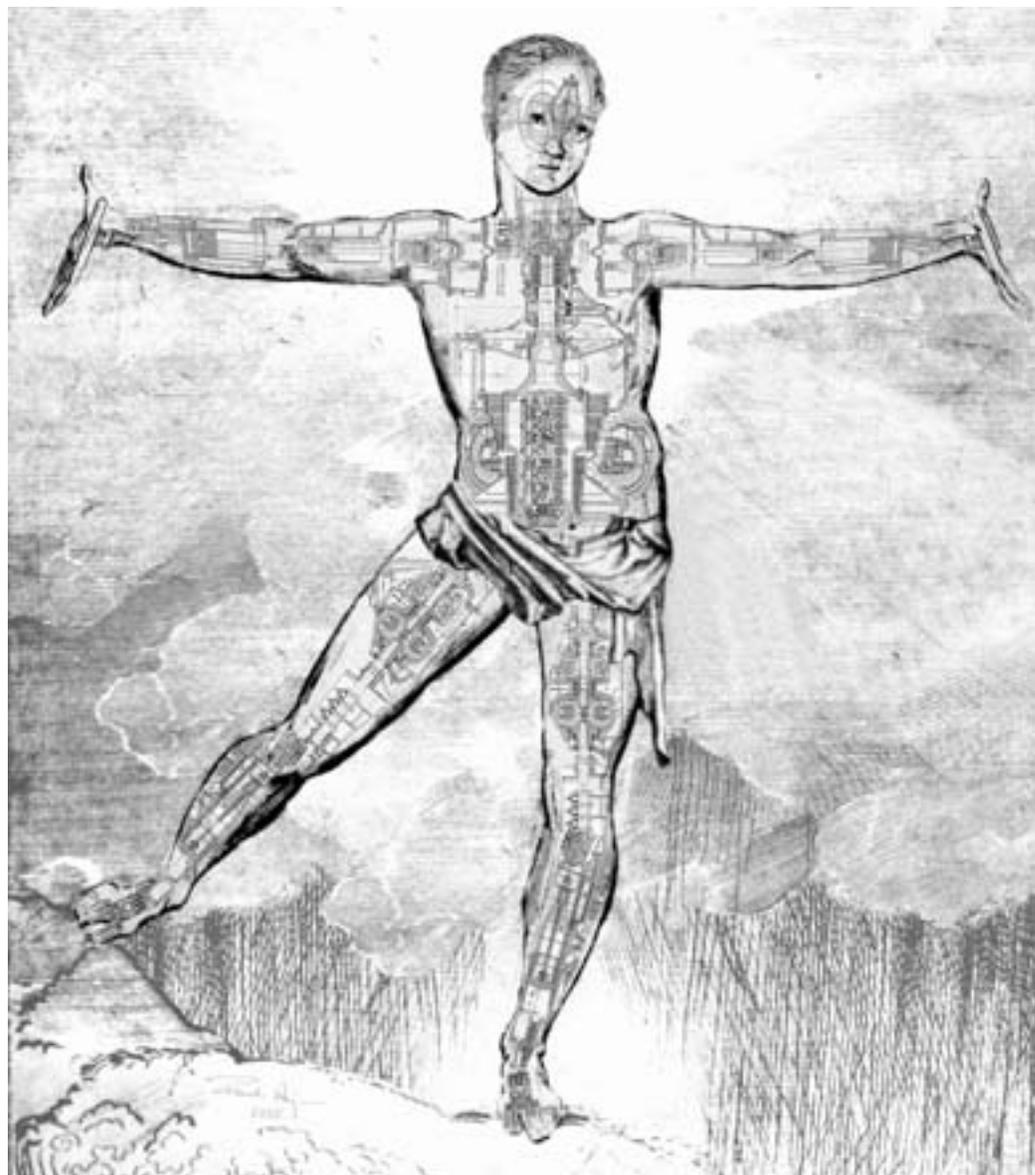
stand a variety of conditions, mechanical wear, changes in weather, gravity and even the amount of cosmic irradiation of the earth have been known to affect performance in complex ways. The same is true for humans. The 'human as gene machine' model is conceptually useful in that it emphasises the complexity of biological systems and the inter-relatedness of the parts (genes), explains how malfunctioning can occur if one or more of the parts is defective (gene variant) or becomes inoperative (mutation), provides for key links between the environment (diet, stress, infectious agent) and the

**By Dr Mark Schena  
and Dr William  
Greene**

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**Figure 1**

Human 'gene machine' modelled as a mechanical device. Artistic rendering of a human being depicted as a mechanical device, with the 25,000 genes in the human body represented as mechanical components. Artwork commissioned from Paul Haje



proper functioning of the machine, and allows for remediation through the use of pharmaceutical compounds that improve the system by altering the output or function of the genes. The gene machines model in the context of drug therapy illustrates how biological systems, genetics and environmental factors collaborate to determine therapeutic outcomes (Figure 2).

### **Biological complexity**

The most recent data indicate that the human genome contains approximately 25,000 genes<sup>1</sup>, with major gene families encoding proteins of regulatory and signalling activity. Popular drug targets such as kinases, phosphatases, G-protein coupled receptors, nuclear hormone receptors and oxyge-

nases, which mediate exquisitely complex signalling hierarchies, comprise more than 1,000 of the 25,000 genes in the human genome. The enormous genetic and biochemical complexity of a human being can be illustrated using simple combinatorial mathematics. In simplified form, one can model a gene as having two expressed states ('on' and 'off'). This binary 'on/off' model can also be applied to proteins that assume regulatory or non-regulatory states and so forth. Using a binary model, a single human cell expressing only 100 genes could assume  $2^{100}$  ( $1 \times 10^{30}$ ) different physiological states. With a repertoire of 25,000 genes operating in more than  $10^{13}$  cells, it should be obvious that the human 'gene machine' possesses an essentially infinite number of possible physiological states

(although a very limited number of such states are optimised at any one time for the state of growth and development of the organism). With respect to drug development and toxicity, the enormous complexity and diversity of human beings mandate approaches that can identify important variants and states. Deployment of a technology such as microarrays that has sufficient 'bandwidth' to analyse this biological complexity on a genomic scale has enormous potential to reduce what is essentially incomprehensible complexity, to a set of manageable data useful for making scientific, clinical and business decisions.

The genomic biological view is perhaps somewhat difficult to assimilate in a pharmaceutical environment, where so much emphasis is placed on single targets. Certainly a complete understanding of drug target structure and function is a pre-requisite for successful drug development. But the 'target is king' mentality greatly oversimplifies and perhaps blinkers one to the fact that drugs work at the genomic (proteomic) level in the context of complex genetic variability and environmental influence. It might actually be more efficient and effective to initiate the drug discovery process at the genomic level and then work backwards to studying the primary target once promising compounds have been identified. In the long run, only a holistic biological view affords the proper perspective in terms of designing and administering safer and more effective drugs. The pharmaceutical and biotechnology industries are obviously embracing this concept, but perhaps not aggressively enough.

### Genetic components

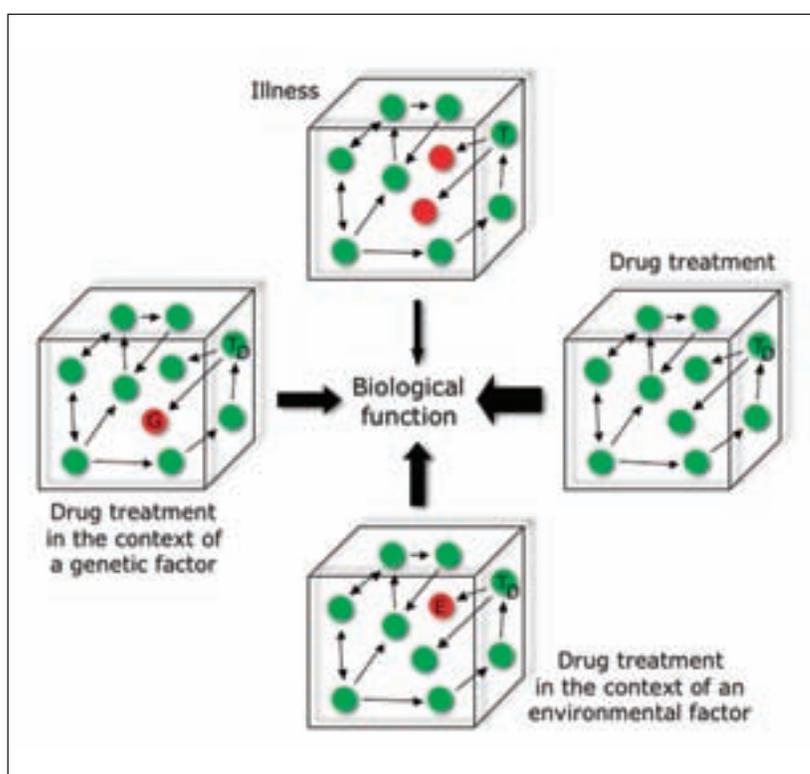
Current estimates based on genomic sequencing and genotyping indicate that human beings share approximately 99.9% sequence identity at the nucleotide level<sup>1</sup>. At first glance, this would seem to suggest that all humans are essentially identical genetically, except for the fact that the genome comprises approximately  $2.9 \times 10^9$  nucleotides. A 0.1% polymorphism rate across 2.9 billion nucleotides would therefore on average give rise to 30 million sequence variants from one person to another, the most important class of which is the single nucleotide polymorphisms (SNP). SNPs that occur in coding regions can significantly alter protein function. Polymorphisms that alter the function of protein involved in drug binding (targets), side reactions ('off-targets'), or drug metabolism, can exert a major influence on drug efficacy and toxicity.

A good example of the importance of understanding patient genotype as a pre-requisite for

optimal drug treatment can be seen in recent advances in *cytochrome P450* (CYP) genetics and diagnostics. The complete sequence of the human genome confirms that the CYP genes comprise a family of approximately 50 genes, important members of which encode iron-based monooxygenases expressed in the liver. CYP liver enzymes use atmospheric oxygen to catalyse the breakdown of foreign compounds such as caffeine, codeine, steroid hormones, organic solvents, antibiotics, antidepressants, non-steroidal anti-inflammatory drugs (NSAIDs and other exogenous compounds and medicines. Patients with fully active CYP enzymes metabolise drugs much more quickly than patients bearing SNPs that impair CYP enzyme function ('slow metabolisers'). Recent estimates suggest that as much as 40% of all P450-dependent drug metabolism is carried out by enzymes encoded by polymorphic genes<sup>2</sup>. The *CYP2C19* gene, for example, contains eight common alleles, seven of which encode inactive P450 enzymes. The *CYP2C9* gene, another important CYP family member that catalyses the turnover of the NSAID Cyclooxygenase-2 (COX-2) inhibitor known as Celecoxib, is also polymorphic. It stands to reason that the 100mg and 200mg recommended daily dosages of Celecoxib (Celebrex®) prescribed to treat osteoarthritis (OA) and rheumatoid arthritis (RA), respectively, may result in very different

**Figure 2**

Molecular model illustrates how genes, genetics and environment collaborate to determine therapeutic outcomes. A human being (square box) contains 10 genes (coloured spheres) that interact (arrows) to impart biological function. An illness (upper box) results from the malfunction of two genes (red spheres) that reside downstream of a drug target (T). The vast majority of individuals (right box) respond normally to drug treatment (D), which restores normal biological function (thick arrow). In rare but important cases (lower and left boxes), drug treatment only partially restores biological function (medium arrows) because genes work in the context of environment cues (E) and genetic variation (G)



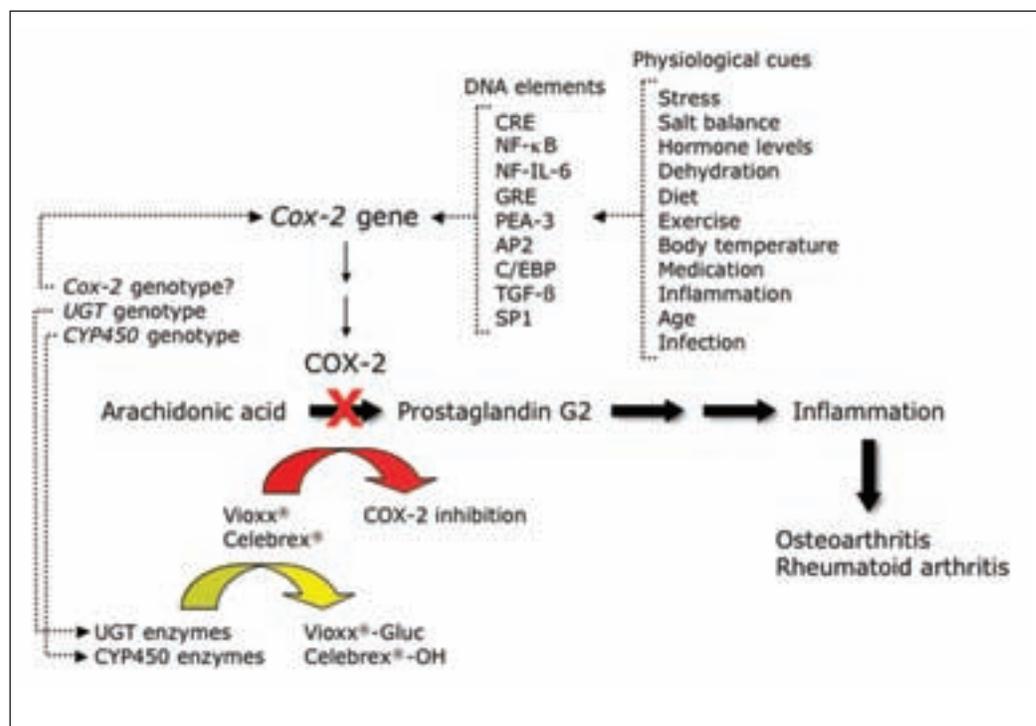
**Figure 3**

Schematic illustration depicts the complexity of COX-2 inhibitor action. Cyclooxygenase-2 (COX-2) catalyses the conversion of Arachidonic acid to Prostaglandin G2, triggering an inflammation response (thick arrows) leading to Osteoarthritis (OA) and Rheumatoid arthritis (RA). COX-2 selective non-steroidal anti-inflammatory drugs such as Vioxx® and Celebrex® bind to the COX-2 enzyme and inhibit its function (red arrow), which slows the inflammation pathway (red X) and ameliorates the symptoms of OA and RA. Numerous physiological cues (eg stress and salt balance) working through known or putative DNA elements (eg CRE and PEA-3 sites) alter COX-2 enzyme levels by activating or repressing *Cox-2* gene expression (thin arrows).

Putative DNA elements include the cyclic AMP (CRE) and glucocorticoid response elements (GRE) as well as binding sites for nuclear factor kappa B (NF-κB), nuclear factor/interleukin-6 (NF-IL-6), polyoma enhancer activator protein 3 (PEA3), activator protein 2 (AP2), CAAT enhancer binding protein (C/EBP), transforming growth factor receptor β (TGF-β), and specificity protein 1 (Sp1).

Polymorphisms in the uracil glycosyltransferase (*UGT*) and cytochrome P450 (*CYP450*) genes produce altered *UGT* and *CYP450* enzymes (dashed arrows), causing COX-2s to be metabolised (eg, Vioxx®-Gluc and Celebrex®-OH) at different rates (yellow arrow) according to patient genotype.

Polymorphisms in the *Cox-2* gene itself (dashed arrow) may also affect the efficiency of COX-2 inhibitors by altering *Cox-2* expression or enzyme structure



serum concentrations from patient to patient depending on *CYP2C9* patient genotype. These individual differences in metabolism, rarely appreciated in standard pharmacokinetic studies on a small number of selected humans during the standard drug development process, can have enormous implications when a drug is launched and used by a wide variety of people with different metabolic activities. The recent launch of *P450* genotyping microarrays by several biotechnology companies offers a means of affordable *P450* genotyping in advance of the administration of COX-2 inhibitors and other drugs.

But the COX-2 story *vis-à-vis* patient genetics is more complex than simply accounting for genetic variability at the *P450* loci. A second important gene family encoding the UDP-glucuronosyltransferase (*UGT*) enzymes appears to provide the major biotransformation function of another widely used NSAID known as Rofecoxib (Vioxx®). Similar to the *CYP* genes, significant genetic variability has been identified in *UGT2B7* and *UGT2B15*, and these polymorphisms may underlie different rates of Vioxx® metabolism in humans<sup>3</sup>. The message here is that patient genetics appear to not only dictate different rates of COX-2 inhibitor turnover in patients, but polymorphisms in the *CYP* and *UGT* genes probably lead to differential rates of metabolism of Celebrex® and Vioxx® depending on whether the patients bear SNPs at

*CYP450*, *UGT* or both. Inasmuch as patients sometimes 'switch' from one COX-2 inhibitor to another, it should be clear that these two important NSAIDs are likely to work differently in patients expressing polymorphic *CYP* and *UGT* enzymes. The COX-2 inhibitors all share the same primary target (COX-2 enzyme), but the body appears to clear Celebrex® and Vioxx® using rather different biochemical pathways. While not emphasised in the scientific literature on COX-2s and NSAIDs in general, it has been widely known among practicing physicians that an individual patient may seem not to 'respond' (gain clinical benefit from) one COX-2, but have excellent effect with another. This observation is likely due in some part, and maybe in very large part, to these individual differences in metabolism.

There is increasing pressure to guide usage and dosage recommendations for Celebrex®, Vioxx® and other important drugs based on patient genetics. To the extent that microarray technology offers an affordable means of consumer-scale genotyping, microarrays appear to be a platform of choice for determining 'drug genotype'. Recent approval of Roche's AmpliChip Cytochrome *P450* test by the Food and Drug Administration (FDA) and the publication of the FDA guidance for drug metabolising genotyping systems<sup>4</sup> are likely to pave the way for a steady stream of genotyping microarray tests for *in vitro* diagnostic use. An

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expanding repertoire of microarray genotyping tests for cytochrome *P450*, *UGT* and other genes involved in drug binding and turnover, as pre-packaged chips and testing services by reference laboratories, are likely to be available in the near future. The use of electronic databases and Internet-based models of data transfer will provide a much-needed real-time link between genotyping information and clinical trials.

### Variable expression

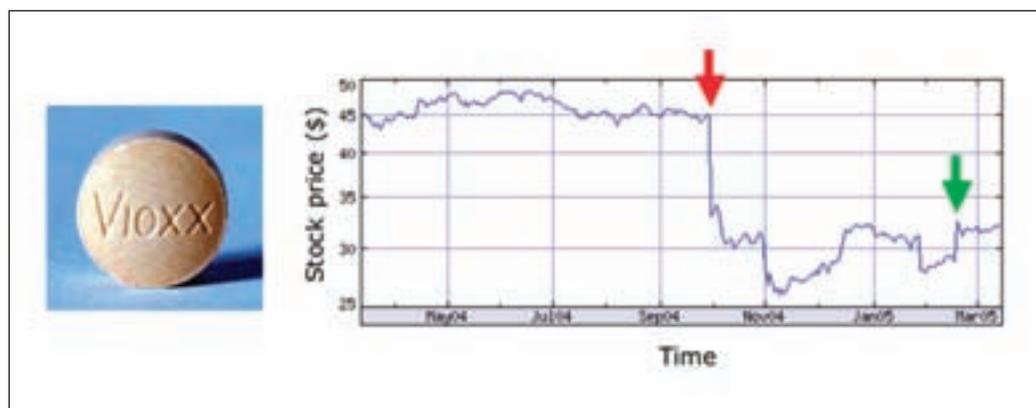
Except for relatively rare somatic mutational events, patient gene sequences are permanently 'hardwired' into the genome as either wild type ('normal') or altered alleles (SNPs). The same is not the case for the expression of genes, which is a highly dynamic process whereby messenger RNA (mRNA) and protein molecules are synthesised from their gene templates. Genes are activated and repressed in response to a plethora of intracellular and environmental stimuli including hormone levels, nutritional status, body temperature, salt balance, stress, bacterial and viral infection, drug therapy and others. Changes in gene expression are mediated by transcription factors, sequence-specific DNA binding proteins that bind in the vicinity of regulated genes and modulate their expression.

The *Cox-2* gene is represented once in the human genome on chromosome 1 and its promoter region contains putative regulatory sites for nuclear factor  $\kappa$ B, interleukin-6, cyclic AMP responsiveness, glucocorticoid receptor, and other factors that regulate *Cox-2* expression<sup>5,6</sup>. The expression of *Cox-2* is regulated through these DNA elements by a complex set of physiological signals including high salt and sugar levels (hyper-

tonicity), dehydration (hypertonicity), nitric oxide (NO), lipopolysaccharides (LPS), arachidonic acid (AA) and other stimuli<sup>7</sup>. Inflammation-induced activation of the *Cox-2* gene leads to elevated levels of the COX-2 enzyme and a concomitant increase in prostaglandin biosynthesis, the biochemical product of COX-2 catalysis. Higher levels of prostaglandins produce the pain and swelling associated with osteoarthritis and rheumatoid arthritis. NSAIDs such as Celebrex® and Vioxx® inhibit the COX-2 enzyme which reduces prostaglandin levels, conferring the anti-inflammatory, analgesic and antipyretic effects of these drugs. Given that multiple cellular factors modulate *Cox-2* expression, the effectiveness of COX-2 inhibitors is expected vary depending on the idiosyncrasies of patient physiology. The complex role of genetic and environmental determinants in COX-2 inhibition (Figure 3) calls into question the current practice of prescribing fixed dosages of these agents to a heterogeneous patient population.

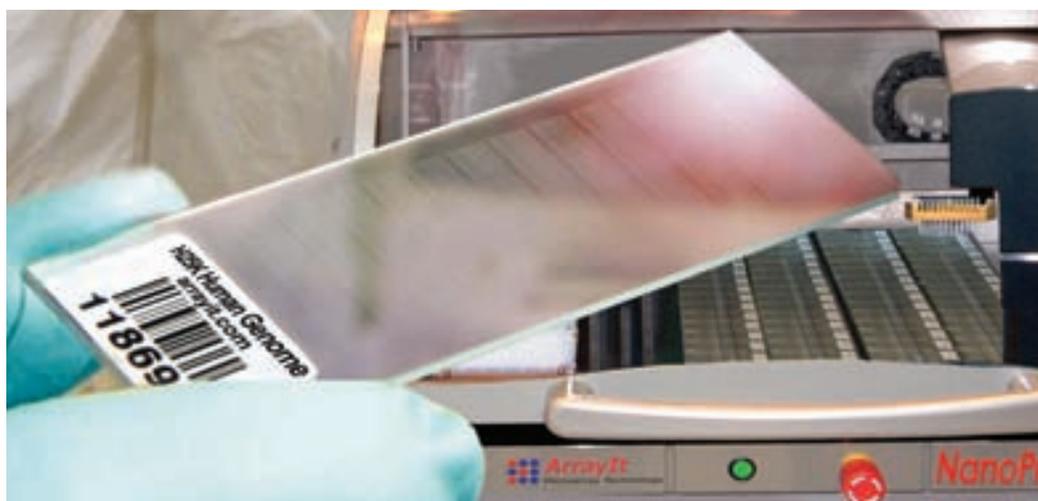
### COX-2 inhibitor toxicity

NSAIDs are the most widely prescribed drugs in the world, with an estimated 100 million users of Celebrex® and Vioxx® since the drugs were introduced on the market in 1999. In the United States alone, sales of Celebrex® and Vioxx® were approximately \$5 billion and \$1.8 billion, respectively, representing nearly 3% of the \$235 billion US prescription drug market. After a recent three-year colon cancer study showed a slight increase in the risk of heart attack and stroke in patients taking Vioxx®, Merck & Company, Inc voluntarily pulled Vioxx® off the market in the fall of 2004, causing an immediate loss of \$28 billion in the



**Figure 4:** Shown is the financial impact of removing a COX-2 inhibitor from the commercial market. (Left) Photograph shows a 25mg tablet of Vioxx®. (Right) Graphic represents the stock price of pharmaceutical giant Merck & Company, Inc (ticker symbol MRK) taken from Yahoo Finance (finance.yahoo.com), with the stock price (US dollars) charted over a one-year period from March 2004 to March 2005 (Time). Merck voluntarily withdrew Vioxx® on September 30, 2004 (red arrow) and the FDA cleared the potential return of Vioxx® to the market on February 18, 2005 (green arrow)

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**Figure 5**  
Shown is a photograph of a whole human genome microarray. The H25K microarray, shown unprocessed after printing, contains 60-mer oligonucleotides derived from 25,509 fully authenticated human genes. The photograph was provided courtesy of the ArrayIt Life Sciences Division, TeleChem International, Inc. (Sunnyvale, California, USA)

company's market value (Figure 4). The Merck stock price is recovering slowly, after the company received FDA permission to place Vioxx® back on the market with a strict 'black box' drug safety warning. Pfizer's widely used COX-2 inhibitors Celebrex® and Bextra® remain on the market, though the safety of both compounds is being studied intensively in view of the fact that Vioxx®, Celebrex® and Bextra® share nearly identical chemical structures. How dangerous are COX-2 inhibitors?

The seriousness of COX-2 inhibitor cardiovascular toxicity, coupled with the widespread use of these drugs and the pervasiveness (and persuasiveness) of an aggressive news media focused on controversy, portray a rather negative portrait of these important therapeutics, and certainly it is important to embrace safety concerns with candour and compassion. But the numbers and the clinical data deserve rigorous analysis. According to one study, the incidence of heart attack and stroke was 0.45% higher in colon cancer patients taking Vioxx® for longer than 18 months compared to a control group taking a placebo<sup>8</sup>. At the very worst, this means that Vioxx® is probably safe for >99.5% of the population and the drug may actually be quite a bit safer than this. For example, it may be that patients predisposed to colon cancer are also predisposed to cardiovascular complications when taking Vioxx®. The connection between cancer and cardiovascular complication is tenuous (at least with what we know today), but the human 'gene machine' is very complex and the interconnectedness of the cellular signalling pathways is relatively poorly understood at the genomic level. It may also be that Vioxx® slightly increases the rate of cardiovascular dis-

ease, but only in patients taking the drug for prolonged periods or at elevated dosages. It may well be that Vioxx® is entirely safe if taken at recommended dosages either periodically or for less than one year. What is needed is a diagnostic test sufficiently powerful to predict COX-2 inhibitor toxicity in advance of heart attack or stroke. More generally, a diagnostic that could be used to help physicians make more evidence-based decisions on risk/benefit ratios for individual patients would be transformative in medicine.

### Whole genome microarrays

Nearly a decade after the first microarray publications emerged<sup>9-14</sup>, the technology has advanced to the point where single 'chips' can be used to quantitatively monitor expression of all 25,000 genes in the human genome (Figures 5 and 6). The basic design of whole genome microarrays is similar to the more modest original assays, though the technical details have changed. Whole genome microarrays are manufactured using either *in situ* synthesis or post-synthesis deposition (Table 1) to produce microscopic collections of oligonucleotides arranged in an orderly fashion on glass. Oligonucleotide microarrays containing gene-specific targets are hybridised with labelled probes derived from human messenger RNA, washed to remove unbound probe, and scanned to detect the fluorescent signals produced by the sequence-specific hybridisation events. The images are quantified to 'extract' the gene expression values and expression data are assembled into databases for data mining and modelling. Examination of a large number of human samples typically provides a clear 'expression signature', several dozen to several hundred genes that are activated or repressed

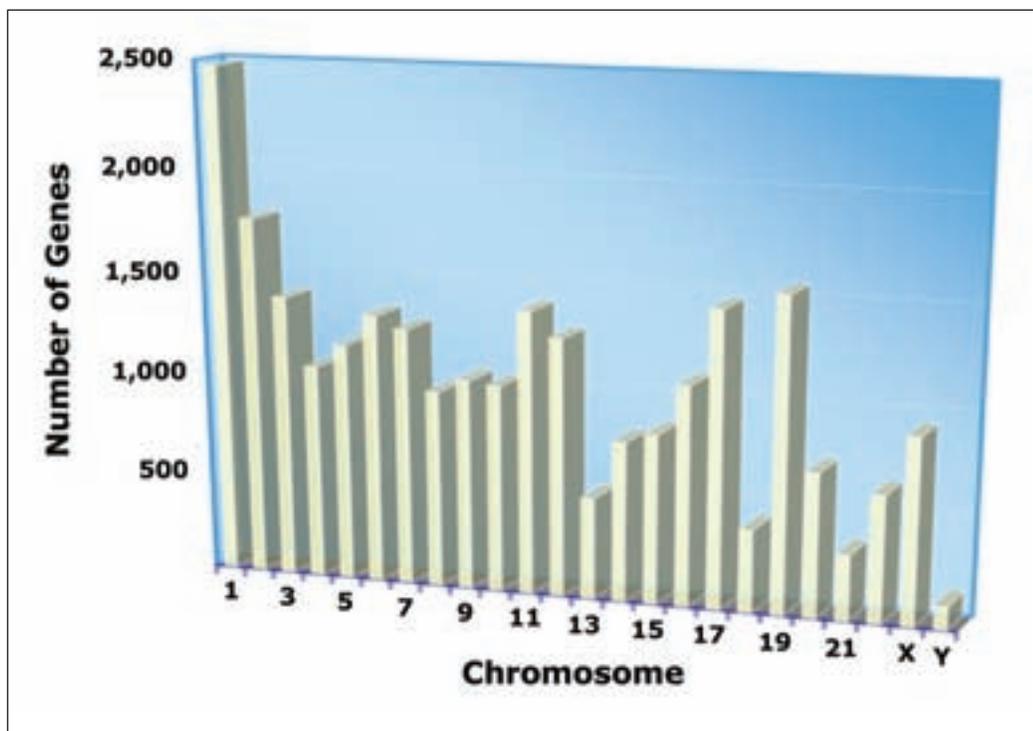
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in a physiological state. The success sub-genomic microarray experiments using celecoxib (Celebrex®) and rofecoxib (Vioxx®) as drug treatments<sup>15-18</sup> indicate that whole genome microarray analysis of a large number of COX-2 inhibitor patients is likely to furnish extremely valuable data.

The appeal of whole genome microarray expression analysis is that the assay is likely to provide a functional diagnostic of COX-2 inhibitor toxicity that is inclusive of many if not all of the factors that affect toxicity including patient genotype (eg *CYP450* and *UGT*), environmental factors (eg diet, stress), patient behaviour (eg dosage non-adherence), and other complexities. The whole genome microarray provides the ultimate 'end point' measurement of the entire human genome in a single test. A COX-2 inhibitor toxicology (risk) profile is expected to emerge in patients at risk for stroke and myocardial infarction in advance of these serious cardiovascular complications. A microarray-based expression profiling test could be offered to patients taking physician-prescribed Vioxx®, Celebrex®, and Bextra® as prolonged treatments for OA and RA.

In the wake of revelations over COX-2 inhibitor

toxicity, good science and medicine suggest that we see this as an opportunity to fine tune the drug discovery process. Genotyping and gene expression microarray assays will likely provide SNP and expression signatures that will be useful in determining patient compatibility with COX-2 inhibitors and other drugs. But the extent to which lemons can be made into lemonade in this case hinges on whether the pharmaceutical industry sees this as the shot over the bow that it is. Vioxx® is back on the market and the dust seems to be settling, but Merck is hardly out of the woods and Pfizer needs to be (and likely is) sweating. The liability associated with toxic drugs is real, and it is in the interest of nobody to see the pharmaceutical enterprise take a direct hit. COX-2 inhibitors are good medicines and they should be made as safe as possible, if for no other reason than for the least defensible reason of all – that making them such would be very good business. More generally, a medical-industrial enterprise that is continually punished post hoc for innovation and risk-taking will simply cease to add value. New technologies such as whole-genome analysis provide the industry with both a tool to refine drug discovery and development, and a weapon with which to fight back.



**Figure 6:** Shown is a graphical representation depicting the number of human genes represented from each chromosome on a whole human genome microarray. The number of genes (X axis) is plotted as a function of human chromosome number including the 22 autosomes and two sex chromosomes (Y axis). The total number of human genes represented is 25,509. Data for the H25K Human Genome Microarray were provided courtesy of the ArrayIt Life Sciences Division at TeleChem International, Inc (Sunnyvale, California, USA)

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**Table 1:** Shown is a list of the current commercial providers of whole human genome microarrays for expression profiling and other genome-wide applications

COMPANY INFORMATION	PRODUCT NAME	METHOD OF MANUFACTURE	DESCRIPTION
Affymetrix, Inc, Santa Clara, CA, USA	Human Genome UI33 Plus 2.0 Array	<i>In situ</i> synthesis by photolithography	Multiple features per gene consisting of 1,300,000 25-mer oligonucleotides derived from expressed sequence databases
Agilent Technologies, Inc, Palo Alto, CA, USA	44K Whole Human Genome Microarray	<i>In situ</i> synthesis by ink-jet printing	Single feature per gene containing 44,000 60-mer oligonucleotides derived from expressed sequence databases
Amersham Biosciences, GE Healthcare, Piscataway, NJ, USA	CodeLink Human Whole Genome Bioarray	Deposition by contact printing	Single feature per gene containing 54,841 long oligonucleotides derived from expressed sequence databases
Applied Biosystems, Foster City, CA, USA	Human Genome Survey Microarray V2.0	Deposition by contact printing	Primarily single feature per gene design containing 32,878 60-mer oligonucleotides derived from the Celera Genomics human genome database
ArrayIt Division, TeleChem International, Inc, Sunnyvale, CA, USA	H25K Whole Human Genome Microarray	Deposition by contact printing	One spot-one gene design containing 26,304 60-mer oligonucleotides derived from a complete human genome sequence

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*Dr Mark Schena received his BA in Biochemistry from UC Berkeley (CA, USA), his PhD from UCSF, and conducted postdoctoral research with Dr Ronald Davis in the Stanford Biochemistry Department. Dr Schena and his Stanford colleagues published the first paper on microarrays in*

*Science magazine in 1995, and since then Dr Schena has lectured and published extensively on the subject.*

*Dr William Greene is a General Partner at MPM Capital, the world's largest dedicated healthcare venture capital firm. Prior to joining MPM, Dr Greene was a Senior Clinical Scientist and Epidemiologist at Genentech with development, regulatory and drug safety responsibilities. He received his MD from UCSF, clinical training and research at Yale, and did further research work at the NIH. He is currently an Assistant Professor of Medicine at UCSF and serves on the boards of several private companies.*