TITLE:

Cardiovascular Safety in Drugs Development: a Role for Endothelial Function Tests

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ABSTRACT

As drug development becomes a long and demanding process, it might also become a barrier to medical progress. Drug safety concerns are responsible for many of the resources consumed in launching a new drug. Despite the money and time expended on it, a significant number of drugs are withdrawn years or decades after being in the market. Cardiovascular toxicity is one of the major reasons for those late withdrawals, meaning that many patients are exposed to unexpected serious cardiovascular risks. It seems that current methods to assess cardiovascular safety are imperfect, so new approaches to avoid the exposure to those undesirable effects are quite necessary. Endothelial dysfunction is the earliest detectable pathophysiological abnormality, which leads to the development of atherosclerosis, and it is also an independent predictor for major cardiovascular events. Endothelial toxicity might be the culprit of the cardiovascular adverse effects observed with a significant number of drugs. In this article we suggest the regular inclusion of the best-validated and less invasive endothelial function tests in the clinical phases of drug development, in order to facilitate the development of drugs with safer cardiovascular profiles.

THE CURRENT PROBLEM OF DRUG DEVELOPMENT

The process of drug development is complex, time-consuming and costly. After a preclinical phase -which could take more than 4 years- the approval of an investigational new drug application to the Food and Drug Administration (FDA) is required, and only then the clinical phases I, II, and III are ready to be started. Following their completion, FDA can approve or reject the new drug, or can also request for more studies before a decision. The entire process can rarely be completed in less than 12 years [1]. Moreover, for every 5000 compounds that initiated the preclinical phase only one will reach the market [2], and the current cost of developing a new drug is continuously growing, nowadays it is estimated to exceed US $990 million [3].

A negative consequence of this challenging path has been the significant decline in the number of new drugs submitted to the FDA, not in parallel with the increased number of relevant biomedical discoveries. This wide gap between basic research and clinical application could impede innovation and limit the number of therapies available for several diseases [4]. A concern about this problem has arisen, and some strategies to improve this critical path from laboratory concept to commercial product are being developed. In the case of medical products performance is evaluated as safety and effectiveness, so one of these requests is for creating new tools to demonstrate them in a more accurate, faster and lower-cost approach [4-6].

Toxicity in drug development

In the past, adverse pharmacokinetic and bioavailability were responsible for the majority of attrition in drug development; currently these reasons contribute less than

10%, and the primary causes are the lack of efficacy (30%) and toxicity (30%). These problems are considerable contributors to the elevated cost of the process, as tend to be recognized in latter stages (phases II and III) or even after marketing [7]. In addition, it is known that over 90% of the market withdrawals are due to drug toxicity, which can lead to a huge expenditure of money and time [8]. For example, it was estimated that the financial and legal cost of withdrawing rofecoxib cost Merck around US $28 billion [9].

Although safety issues are a cause of delay and discontinuation during the process, and even a possibility of eliminating unnecessarily potential candidates exists, it seems neither practical nor ethical to simply lower the safety standards, as some people have proposed. We expect marketed drugs to have a well-understood safety profile and a positive benefit/risk balance. However, despite the major relevance of safety assessment, there have been little changes over the years in the traditional tools used for it [4-6]. But even more, some concerns are present regarding a higher likelihood, compared to previous decades, of unanticipated safety problems once the drug is approved [10], and about an inexplicable deferral in removing those drugs from the market following the detection of severe side effects [11, 12].

The determinants for these safety deficits are diverse and not unique. Among the reason we could include: the lack of specificity to predict the adverse effects in humans that classical animal toxicology may have; the narrow spectrum of patients profiles enrolled in clinical phases that can differ importantly from the population that will receive the treatment after the approval; the incapacity to detect during the short follow-up of clinical trials those side effects which appear in a very late stage; and the fact that

serious adverse drug reactions (ADRs) are often so rare that a huge number of individuals are required to identify them [4, 5, 8].

CARDIOVASCULAR SAFETY

The high number of treatments developed for cardiovascular diseases may also have undesirable negative effects on the same anatomy where they act. Besides, the cardiovascular system has proven to be particularly sensitive to a large variety of interaction with drugs prescribed for a different therapeutic indication. In result, the cardiovascular system is the most frequent sites of ADRs, and cardiovascular safety is the major cause for drug discontinuation at all stages of drug development in United States [13].

Unfortunately, its leadership does not seem to have changed during last decades. Some years ago, Lasser et al. [14] analyzed the period from 1975 to 2000 and found 81 major changes to drug labeling in the Physicians' Desk Reference, including the addition of one or more black box warnings or drug withdrawal. Cardiovascular (21%) and hepatic (19%) toxicities were the main culprits of those changes. Afterwards, Schuster et al. [8] studied the reasons for drug withdrawals from European and American markets between 1992 and 2002. They collected a total of 16 drugs withdrawals, 94% of them due to safety problems. Again cardiovascular (40%) and hepatic (27%) toxicities were the principal contributors for market discontinuation. And more recently, concordant data coming from two pharmaceutical companies (DuPont-Merck and Bristol-Myers-Squibb) during 1993-2006 [15], describe how the most frequent organs or tissues affected by toxicity are the cardiovascular system (27.3%) and the liver (14.8%).

Mechanisms and evaluation of cardiovascular toxicity

A chemical compound may impair the cardiovascular system performance through three particular mechanisms: inducing direct myocardial injury, promoting proarrhythmic changes and/or altering the vascular integrity and tone. The consequences of these insults depend on both the drug (type, dose, time of exposure) and the patient (age, gender, race, healthy status, concomitant treatments). Based on that, their magnitude may be quite diverse: from cardiovascular death or severe irreversible injuries (e.g. myocardial or cerebral infarction), to symptomatic or asymptomatic reversible effects (e.g. deterioration of ventricular ejection fraction, non-lethal arrhythmias), or pathophysiological alterations that could predispose the patient to future cardiovascular events (e.g. hypertension, arrhythmogenic or thrombogenic substrates) [16, **17**].

The current approaches for assessing cardiovascular safety of new drugs, and their particular limitations, have been recently and extensively reviewed in the literature. As a brief summary, the direct drug-induced myocardial injury can be evaluated from the examination of isolated cultured cardiomyocytes or histopathological tissue samples from animals, and with the use of different biomarkers (troponins, natriuretic peptides), imagine (echocardiography, magnetic resonance imaging) and invasive techniques (hemodynamic catheterization) **[13, 16-18]**. The proarrhythmic risk, which has received great attention in last years [19, 20], **has been traditionally** estimated with the functional evaluation of the potassium channel (I_{Kr}) responsible of most drug-related long QT syndromes (hERG assay), the study of action potentials in isolated cardiac tissues, and with continuous monitorization of arrhythmias and electrocardiographic intervals

(electrocardiograms, telemetry); **however more recently a new cardiac proarrhythmia safety paradigm has been proposed, it is labeled the "Comprehensive In vitro Proarrhythmia Assay" (CiPA) and includes in silico predictive modelling of cellular electrophysiological effects [21].**

But, as previously recognized [13, **17,** 22], little efforts (e.g. lipid profile, inflammatory markers, blood pressure monitoring) have been done to analyze the drug-related pathophysiological alterations that could produce middle-long term effects in the cardiovascular system. Most of the interest has been exclusively focused on the acute and proarrhythmic consequences of the new compounds, especially their risk of QT prolongation.

The present of cardiovascular toxicity

We have performed a search for the marketed drugs withdrawn by FDA during a 5 year period (2005 - 2010) [23]. At our knowledge, there have been at least 5 withdrawals related to an associated increased cardiovascular risk (Table 1). In addition, 12 safety alerts concerning increased cardiovascular risk associated with the use of various compounds have been published in the same period (Table 2).

In agreement with the data reported by Lasser et al [14], in both tables we can visualize how the majority of ADRs are discovered years, even decades, after the drugs are on market. Likewise, it should also be noted that QT prolongation is still one of the most frequent reason for cardiovascular safety alert, but clearly, it is not the only responsible.

Remarkably, as a good demonstration of the uncertain current times, even acetaminophen -which is traditionally considered the drug of choice for pain relief in patients with cardiovascular disease due to its theoretical cardiovascular safety-, has recently shown to significantly increase heart rate and blood pressure compared to placebo [24], in similarity with many nonsteroidal antiinflammatory drugs (NSAIDs) [25].

The relevance of the cardiovascular safety assessment in the process of drug development is never sufficiently highlighted. With the present delays in detecting this toxicity, we are exponentially increasing the cost, being at risk of ending the research in new molecules, and so taking away the hope for thousand of patients. For instance, it has been suggested [26] that one of the Pfizer's reasons to interrupt development of new drugs in cardiovascular area were the results (increased deaths and cardiovascular events) in a phase III clinical trial with torcetrapib [27].

At this point, it is reasonable to conclude that cardiovascular toxicity continues to be underestimated at drug's market launch, and that current methods to select drugs with a proper cardiovascular safety profile are still inaccurate and insufficient. So, we have an imperative need for new approaches to help us to deliver cardiovascular safe drugs at acceptable times and reasonable cost, avoiding patients to exposure unnecessarily to deleterious cardiovascular ADRs.

Table 1. Drugs withdrawn from the market based on cardiovascular safety concerns between 2005 and 2010

Drug Name	Brand Name	Company	Approval Date	Drug Use	Withdrawal Date	Withdrawal Reason
Propoxyphene	Darvon	Xanodyne Pharm	08/16/1957	Pain	11/19/2010	Increased risk of abnormal heart rhythms (PR and QT prolongation) [28]
Sibutramine	Meridia	Abbott	11/22/1997	Obesity	10/08/2010	Increased risk of heart attack and stroke [29]
Pergolide	Permax	Lilly	12/30/1988	Parkinson's disease	03/29/2007	Increased risk of heart valve disease [30]
Tegaserod	Zelnorm	Novartis	07/24/2002	Irritable bowel syndrome	03/30/2007	Increased incidence of cardiovascular ischemic events [31]
Valdecoxib	Bextra	Pfizer	11/16/2001	Pain	04/07/2005	Increased risk of cardiovascular events [32]

Table 2. Safety alerts concerning cardiovascular risk published between 2005 and 2010

Drug Name	Brand Name	Company	Approval Date	Drug Use	Safety Alert Date	Safety Alert Reason
Dolasetron	Anzemet	SanofiAventis	11/09/1997	Nausea, vomiting	17/12/2010	Increased risk of QT prolongation and Torsades de Pointes [33]
Saquinavir	Invirase	Roche	12/06/1995	HIV	10/21/2010	Potential change in the electrical activity of the heart (PR, QT) [34]
GnRH Agonists	Lupron Eligard Trelstar Zoladex	Abbott SanofiAventis Watson AstraZeneca	04/09/1985 01/23/2002 05/15/2000 12/29/1989	Prostate Cancer	10/20/2010	Increased risk of diabetes and certain cardiovascular diseases (heart attack, sudden cardiac death, stroke) [35]

Rosiglitazone	Avandia	GSK	05/25/1999	Diabetes Mellitus	09/23/2010	Increased risk of cardiovascular events, such as heart attack and stroke [36]
Fosamprenavir	Lexiva	GSK	10/20/2003	HIV	12/03/2009	Increased risk of myocardial infarction and dyslipidemia [37]
Haloperidol	Haldol	Johnson and Johnson	04/12/1967	Agitation	09/17/2007	Increased risk of QT prolongation and Torsades de Pointes [38]
Thiazolidinediones	Avandia Actos	GSK Takeda	05//25/1999 07/15/1999	Diabetes Mellitus	08/14/2007	May cause or exacerbate heart failure [39, 40]
Methadone	Dolophine	Roxane	08/13/1947	Pain	11/27/2006	Increased risk of QT prolongation and Torsades de Pointes [41]
Imatinib	Gleevec	Novartis	05/10/2001	Chronic Myeloid Leukemia, Gastrointestinal Stromal Tumors	10/19/2006	May cause or exacerbate heart failure [42]
Trastuzumab	Herceptin	Genentech	09/25/1998	Breast cancer	08/31/2005	Cardiotoxicity (decreased LVEF, increased risk of heart failure and cardiac death [43]
COX-2 Selective and Non-Selective NSAIDs	Various	Various	Various	Pain	04/07/2005	Increased risk of cardiovascular events [44]
Bevacizumab	Avastin	Genentech	02/26/2004	Colon and rectum cancer	01/05/2005	Increased risk of arterial thromboembolic events, including myocardial infarction and angina [45]

WHY TESTING ENDOTHELIAL FUNCTION?

The endothelium is a large homeostatic organ that plays a major role in cardiovascular physiology and disease. Its structure might be seen as a simple cell monolayer lining the entire vascular lumens, but its functions are much more complex and relevant. Through the synthesis and release of several bioactive substances, primarily but not only nitric oxide (NO), it regulates vascular tone, and prevents vessels wall inflammation, smooth muscle cell proliferation, and thrombosis [46-48]. Several pathological circumstances may induce functional and structural alterations. The resultant endothelial dysfunction involves a systemic disorder that comprises the production of vasoconstricting and prothrombotic factors, the expression of adhesion molecules, and the impairment of the normal repair mechanisms. At present, we have available a wide range of invasive and non-invasive methods to assess this endothelial activation in vivo and in vitro [48, 49].

Clinical relevance of endothelial dysfunction and utility of endothelial function tests

All cardiovascular risk factors, such as diabetes mellitus, hypercholesterolemia, hypertension, obstructive sleep apnea and smoking [50-54], have shown to impair endothelial function. Although contradictory data exist [55], it has also been pointed out that endothelial dysfunction might be not only the consequence, but even a pathogenetic mechanism for the onset of some of them [55-59].

Endothelial dysfunction is recognized as one of the factors responsible for initiation and progression of atherosclerosis [60, 61], both for the loss of its protective properties and for the induction of an atherothrombotic substrate [62]. It is an

independent predictor for future major cardiovascular events [63], as it also contributes to destabilize the plaque, by changing its biology and composition [64], making it more prone to rupture and thus to acute cardiovascular events [65]. Besides, heart failure is a casual factor for endothelial dysfunction [66] and, at the same time, it is linked to worse outcomes and high mortality in patients with heart failure [67, 68].

Currently, endothelial tests are being used for several applications. They are excellent approaches for a better understanding of the mechanisms involved in the genesis and progression of many different diseases (i.e. atherosclerosis [69], erectile dysfunction [70], pulmonary hypertension [71], renal insufficiency [72], migraine [73]); they are quite helpful in assessing the changes in endothelial function and clinical markers resulting from exercise [74], dietary [75-77], medical [78-80], percutaneous [81] or surgical interventions [82]; and one of their most promising advantages is their applicability as clinical diagnostic tool for identifying -at earlier stages- those patients with a high risk of cardiovascular events, in order to initiate or intensify the proper treatments [83, 84].

Drugs and endothelium

It has been observed in animals that endothelial function and structure may result damaged by three different drug-related mechanisms [85]: a) direct endothelial cell toxicity, through interactions with molecules expressed on the cell membranes; b) an increase in blood flow-induced shear stress, generated from prolonged vasodilatation or a marked increment in regional blood flow; c) an immune-mediated injury. Following any of them, there is a common endothelial activation –quite similar to the observed with the

major cardiovascular risks- that comprises synthesis and release of proinflammatory cytokines, upregulation of adhesion molecules, T cell and complement activation, and autoantibodies production. All these actions result in vessel wall inflammation, leading to an increase intimal permeability, membrane damage, intimal hyperplasia and cell death [86].

The number of marketed drugs with proven arterial toxicity in animals is not negligible [85], and it would be possible that the list of drugs that induce endothelial dysfunction in humans was longer in case we tested all compounds. We will just mention below some of the most noticeable interactions, due to both their recent description and clinical relevance.

One of the particularly controversial topics in recent years [9, 11, 12] have been the market withdrawal of some cyclooxygenase 2 (COX-2) selective NSAIDs, and the FDA safety alert for the rest of COX-2 selective and non-selective NSAIDs due to potential serious adverse cardiovascular events [32, 44, 87]. While the exact mechanism by which these drugs increase cardiovascular risk is still not fully understood, today we have more clues about the pathological role of endothelial dysfunction on it [79, 88, 89]. COX-2 was thought to be only an inducible enzyme associated with inflammation and pain; but it is easily inducible in endothelial cells by shear stress too [90]. There, COX-2 produces prostacyclin (PGI_2), which promotes vasorelaxation and inhibits platelets activation. One of the postulated mechanisms is that the inhibition of COX-2 will induce the loss of these endothelial PGI_2 cardioprotective effects, leading to the undesirable cardiovascular effects [91].

In the last two decades, oncology is becoming a medical specialty with a highly productive research in new drugs. It is reducing the mortality and morbidity of patients with cancer, but at the same time is revealing a large number of cardiovascular ADRs. This fact may limit the use of some of these compounds, given that many of the signaling cascades inhibited in cancerous cells are also necessary for myocardial and vascular cells survival [92]. The vascular endothelial growth factor (VEGF) is essential for growth and survival of endothelial cells [93], so anti-VEGF drugs (i.e. bevacizumab, lapatinib, sunitinib, sorafenib) are a good paradigm [94].

The idea of raising protective high-density lipoprotein (HDL) seems attractive, as it is known to enhance endothelial function [95]. Therefore the early termination of a phase III clinical trial with torcetrapib [27], a cholesteryl ester transfer protein inhibitor, because of an increased risk of death and cardiac events, was not expected. The real cause of these adverse events is still unclear, but a low increase in the blood pressure and serum aldosterone are unlike to entirely explain the magnitude of the outcomes [96, 97]. The first evidences for torcetrapib-induced endothelial dysfunction in vivo are already published [98].

In chronic kidney disease, oxidative stress and inflammation are associated with impaired activity of the nuclear 1 factor (erythroid-derived 2)-related factor 2 (Nrf2) transcription factor. Bardoxolone methyl is a potent activator of the Nrf2 pathway and was shown to reduce the serum creatinine concentration. However, significantly increased risks of heart failure and of the composite cardiovascular outcome (nonfatal myocardial infarction, nonfatal stroke, hospitalization for heart failure, or death from cardiovascular causes) prompted termination of a

randomized trial in patients with type 2 diabetes and stage 4 chronic kidney disease [99]. It has been seen that through modulation of the endothelin pathway –a potent vasoconstrictor peptide produced in endothelial cells-, bardoxolone methyl may promote acute sodium and volume retention and increase blood pressure in patients with more advanced chronic kidney disease [100].

Moreover, different antipsycotics (haloperidol, risperidone, chlorpromazine and clozapine) have been recently related to cytoxic effects and apoptosis of endothelial cells [101]. This might be one of the reasons for the significantly increased risk for stroke and coronary artery disease observed with the use of second-generation antipsycotics [102].

Endothelial function tests to be integrated in drugs development

It is beyond the scope of our article to provide a description of the tests that are used or that might be applied in the preclinical phases of drugs development. Besides, there are significant concerns regarding the uncertain extrapolation of some induced vascular toxic effects observed in animals to humans [85, 86]. Thus, we will just focus on detailing the evidence that might support, according to our opinion, the introduction of the best validated and less invasive techniques for the assessment of endothelial function in the clinical phases of drug development.

These techniques may be grouped into two categories: those tests that measure the appropriate endothelial respond to increased shear stress (flow-mediated dilatation [FMD] and reactive hyperemia peripheral arterial tonometry [RH-PAT]), and those that

evaluate the production of biomarkers of endothelial damage and repair (asymmetric dimethylarginine [ADMA] and endothelial progenitor cells [EPCs]).

The most widely used non-invasive test is the FMD, which consists in the ultrasound measurement of the changes in brachial artery diameter due to the release of endothelial NO, in respond to the increase in shear stress induced by the inflation and subsequent release of a sphygmomanometer cuff on the distal forearm. This response is depressed in subjects with atherosclerosis and cardiovascular risk factors [49]. The assessment of peripheral endothelial function by FMD is close related with coronary artery endothelial function [103]. FMD is an independent predictor of cardiovascular events in subjects with [104, 105] and without [83] previous cardiovascular disease.

More recently, another non-invasive technique to assess the peripheral endothelial function has been developed. RH-PAT measures the changes in digital pulse volume during a similarly induced reactive hyperemia. In the same way, this digital vasodilatation function is related to multiple traditional cardiovascular risk factors [106] and to coronary microvascular endothelial dysfunction [107]. RH-PAT is also an independent predictor of cardiovascular adverse events [84].

Increased plasma levels of ADMA -an endogenous competitive antagonist of NO synthase that impairs endothelial function- are detected in subjects with cardiovascular risk factors and diseases [108], and are related to coronary endothelial dysfunction [109] and decreased branchial FMD responses [110]. Elevated ADMA levels are an independent predictor of future major adverse cardiac events [111] and all-cause mortality [112].

Endothelial function is related to the number of EPCs as these EPCs are responsible for maintaining endothelial integrity after many of the injuries. An inverse correlation has been shown between cardiovascular risks factors and diseases and the number and function of EPCs [113]. As seen above with the previous three tests, low levels of EPCs are also independent predictors of CAD progression [114] and worse cardiovascular outcomes [115, 116].

CONCLUSIONS

In the present article, we have shown that cardiovascular safety remains a key problem in drug development. Despite the approaches currently used to detect the toxicity along this process, patients continue to suffer severe cardiovascular side effects once the drugs are already in the market. It has been clearly stated that endothelial dysfunction plays a main role in the incidence of future major cardiovascular events, and that endothelial function tests are valuable tools to determinate the endothelial morphologic and functional integrity. Our review support the inclusion of some of these endothelial function tests in the hard process of drug development, for avoiding the undesirable consequences of drug-induced endothelial toxicity.

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