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Predictivity of *in vitro* non-clinical cardiac contractility assays for inotropic effects in humans – A Literature Search.

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Abstract

Adverse drug effects on the cardiovascular system is a major cause of compound attrition throughout compound discovery and development. There are many ways by which drugs can affect the cardiovascular system, including effects on the electrocardiogram, vascular resistance, heart rate and the force of contraction of the heart (inotropy). Compounds that increase the force of contraction of the heart can be harmful in patients with ischemic heart disease, whilst negative inotropes can induce symptoms of heart failure. There is a range of non-clinical *in vitro* and *in vivo* assays used to detect inotropic effects of drugs. We have conducted a literature review of the *in vitro* assays and compared the findings from these with known effects on cardiac contractility in man. There was a wide variety of assays used, ranging from perfuse whole hearts to isolated regions of the heart (papillary muscle, ventricle and atria), which were removed from a number of species (cat, guinea pig, rabbit and rat). We conducted two analyses. The first was investigating the concordance of the findings from the *in*

vitro assays at any concentration with those observed in man (an assessment of hazard identification) and the second was the concordance of the *in vitro* findings at concentrations tested up to 10-fold higher than those tested in the clinic. We found that when used as a hazard identification tool, the available assays had good sensitivity (88%), although the specificity was not so good (60%), but when used as a risk management tool the sensitivity was considerable reduced (sensitivity 58 -70% and specificity 60%).

These data would suggest that the available *in vitro* assays can be used as hazard identification tools for adverse drug effects on cardiac contractility, but there is a need for new assays to better predict the exposures in man that may cause a change in cardiac contractility and therefore better predict the likely therapeutic index of compounds prior to nomination of compounds for clinical development.

1. Cardiac Contractility – A cardiovascular Risk factor.

Compound attrition remains a major challenge for the pharmaceutical industry through out the drug discovery and development process. Even in Phase II and Phase III clinical trials drug-related safety attrition accounts for 28% of all causes of compound failure, second to the lack of efficacy as the major cause of clinical attrition (Arrowsmith & Miller, 2013). An adverse drug effect on the cardiovascular system is one of the major causes of safety-related attrition both in the non-clinical and clinical phases of drug development (Cook *et al.,* 2014). This is despite the introduction of regulatory guidance that mandates the investigation of the effects of all new chemical entities on the cardiovascular system in a non-rodent species (ICH S7A). Interestingly, the core package of

safety pharmacology studies outlined in ICH S7A recommends an investigation of drug effects on blood pressure, heart rate and the electrocardiogram. An investigation of effects on cardiac contractility is only recommended as part of the follow-up/supplementary studies.

Drug effects on the cardiovascular system are complex with potential actions on vascular tone, heart rate, cardiac conduction and the force of contraction of the heart. Furthermore, because of the complex neurohumoral control of the cardiovascular system drugs can have additional indirect effects on different parts of the cardiovascular system. For example, vasodilator mechanisms can cause reflex changes in cardiac function, in the absence of a direct effect on the heart (Miller *et al.,* 1975). Thus in order to characterise drug effects on the cardiovascular system it is important to test for both direct and indirect effects on both the heart and vascular tone.

Myocardial contractility reflects the ability of the heart to contract resulting in a change in force generation, which is independent of pre-load (sarcomere length). This increase in force against a constant after-load results in a greater volume of blood being ejected from the ventricle i.e. an increase in stroke volume. Relaxation of the heart is the process by which the heart muscle actively returns, after contraction, to its initial conditions of load and length and this is referred to as lusitropy. A rapid and complete relaxation is required for cardiac output adaptation to changes in loading conditions, inotropic stimulation and heart rate. Thus it is possible for drugs to affect the active relaxation (lusitropic effects), contraction (inotropic) and rate of beating (chronotropic) of the heart.

Drug induced increases and decreases in cardiac contractility can be harmful depending on susceptible patient populations. Drugs that have a direct positive inotropic effect on the heart will cause an increase in myocardial oxygen demand, which in normal healthy volunteers may be without consequence, but in patients with coronary artery disease such drugs can be associated with a risk of myocardial hypoxia. Cardiac stimulants that act through inhibition of cAMP phosphodiesterases have been shown to increase mortality in patients with severe chronic heart failure (Packer *et al.,* 1991). Compounds that have negative inotropic activity, such as itraconazole, can induce symptoms of heart failure (Ahmad, Singer, & Leissa, 2001). Thus, testing for drug effects on the contractility of the heart needs to take into account the potential patient population, but even in areas of high medical need, such as oncology, untoward effects on the cardiovascular system has become so significant that a new society has been formed to better advance the science in this area of oncology (International Cardiooncology Society, http://icosna.org).

2. Non-clinical methods to detect drug effects on cardiac contractility.

The isolated perfused mammalian heart preparation was established in 1897 by Oscar Langendorff. These preparations are widely used to study the biochemistry of ischemia-reperfusion injury, drug effects on the ECG, heart rate and cardiac contractility (Skrzypiec-Spring, Grotthus, Szelag, & Schulz (2007). The isolated rat, guinea-pig and rabbit hearts have been used to study drug effects on the force of contraction of the heart using either a constant perfusion flow rate or constant perfusion pressure. One of the advantages of the isolated

heart is that the rate of beating can be controlled and thus effects on the force of contraction, independent of rate, can be studied. Furthermore, the preparation enables a direct effect on the heart to be confirmed, as opposed to reflex changes, which may be observed in the intact animal. One of the disadvantages of the Langendorff preparation is that because there is essentially an open perfusion system there is no afterload resistance, unlike in the *in vivo* situation. For a recent detailed review on the Langendorff preparation refer to Bell et al (2011).

In addition to the isolated perfused heart, a range of individual tissue preparations has been studied. Cardiac muscle fibre studies have been utilized for many years to assess maximum upstroke velocity (Vmax) (Grant et al., 1978) as an indication of contractility. In addition the isolated rat and rabbit ventricle are well suited for studying the inotropic effects of drugs (Ito *et al.,* 1996) since the ventricles are the main contributors to inotropic state of the heart *in vivo.* Other preparations include the isolated atria, trabeculae and papillary muscle. The latter is often primarily used to study drug effects on the cardiac action potential and the force of contraction recorded as an additional end point (Honerjäger *et al.* 1986) making the preparation ideal for studying electromechanical coupling of the heart and the impact of drugs thereon.

Drug effects on the force of contraction of the heart have been studied in a wide range of *in vivo* preparations. The main advantages of these approaches are that they allow an assessment of cardiac contractility as one part of the overall haemodynamic profile of a compound, thus establishing an integrated cardiovascular assessment. The main disadvantages is that some assessments of

cardiac contractility can be complex and it can be difficult to differentiate between a direct drug effect on the heart and an effect secondary to a change in vascular tone or heart rate. The preferred approach to assessing a drug effect on cardiac contractility is through the measurement of the relationship between the ventricular pressure versus volume from which the wall stress versus strain relationship of the heart throughout the cardiac cycle can be estimated (Suga, Sagawa and Shoukas 1973). The main advantage of such analysis is that they estimate drug effects on cardiac contractility independent of additional effects on heart rate and also pre- and after-load. However, these techniques are complex and do not easily apply to the routine testing of new chemical entities and therefore a number of alternative techniques have been explored. One of the most common approaches has been to use the maximal rate of pressure increase in the left ventricle during systole (LVdP/dtmax). The introduction of a pressure transducer into the left ventricle, from which LVdP/dtmax is derived, has been used in many laboratory species including telemetered dog, monkey and mini-pig (Markert *et al* 2007) and rats (Sato, Kandori and Sato 1994). Although commonly used as a measure of cardiac contractility, LVdP/dtmax is influenced by heart rate and as such a drug-induced change in heart rate will affect LVdP/dtmax (Markert *et al* 2007). In addition, LVdP/dtmax can be influenced by both pre- and after-load (Hamlin and del Rio 2012), although some authors have suggested that pre-load has minimal effects on LVdP/dtmax (Zimpfer and Vatner 1981).

Other, indirect methods for assessing drug-induced changes in cardiac contractility include the QA interval, the time between the Q-wave of the ECG

and upstroke of aortic pressure (Cambridge and Whiting 1986). Although the QA interval is a useful indicator of cardiac contractility (Norton, Iacono and Vezina 2009) it can be influenced by heart rate (Adeyemi *et al* 2009). Nevertheless, because it is a parameter that is easily derived from blood pressure and ECG recordings it can be used to exclude an effect on cardiac contractility if the test substance has minimal effects on heart rate.

In order to accurately assess the effects of compounds on cardiac function, it is important to mimic the true *in vivo* cardiac muscle mechanics as closely as possible. Length-tension relationship is an important factor when investigating muscle mechanics *in vitro*. The length tension relationship relates to the arrangement of actin and myosin filaments in the muscle fibres. At lower resting lengths there is a complete overlap between the thin and the thick filaments, which interferes with cross-bridge binding to active sites resulting in low tension upon muscle stimulation. Maximal number of cross-bridges can be formed when the sarcomere length is optimal, producing highest tension. Further increase in the sarcomere length results in decrease of tension as the size of the overlap zone and the number of potential cross-bridges is reduced (Shiels and White, 2008).

Length-tension relationships can be extrapolated to the whole heart mechanics. The extent of ventricular filling has a direct relationship with the pressure development in the ventricular muscle during contraction. Frank-Starling law refers to the relationship of the force of ejection/pressure to the degree of ventricular filling/ length of muscle fibre at the end of diastole. Increased

ventricular filling leads to extension of the muscle fibres in the ventricular wall, prestretch, before contraction occurs. This corresponds to the change in muscle/sarcomere length (Sperelakis, 2001).

The choice of *in vivo* end points to assess drug effects on cardiac contractility very much depends on the nature of the experiment and the level of instrumentation that is acceptable. As discussed above QA is a valuable, non-invasive measure, in the absence of marked heart rate changes, whilst LVdP/dtmax appears an acceptable endpoint to most investigators and regulators.

Currently, it is practically challenging to investigate pressure-volume behaviour on muscle mechanics in an *in vivo* setting. Therefore, *in vitro* muscle mechanics can be implemented by applying force-length dynamics. Contractile force is corresponding to the pressure against which the muscle is contracting and length relates to the initial muscle length, which is determined by the ventricular volume before the contraction takes place (Sperelakis, 2001).

3. In vitro testing methods of muscle mechanics

The majority of the information on contractile dynamics has been provided by *in vitro* experimentation. However, during *in vitro* experimentation, muscles are usually restricted and therefore do not behave as they would *in situ*. Generally, only a certain aspect of muscle mechanics is investigated using a specific experimental technique, the results obtained should therefore be evaluated with

caution. For example, studies have used constant muscle length (isometric), constant load (isotonic), constant velocity (iso-velocity) or recently work-loop studies to investigate muscle mechanics.

4. Isometric and isotonic contraction studies

As mentioned before, an isometric testing protocol holds the muscle at a constant length throughout the electrical stimulation and force generation to investigate muscles' ability to produce force. Isometric contraction studies using cardiac muscle preparation have investigated the effects of compounds on muscle performance. For example, doxorubicin-induced effects on the isometric contractile force were investigated in the human atrial trabeculae (Montaigne et al., 2011) and rat papillary muscles (Gharanei et al., 2012), which showed doxorubicin-induced reduction in the contractile force. One of the major limitations of isometric contraction methods is that it does not reflect the *in vivo* muscle characteristic. It is very rare for any muscle to remain at a constant length throughout the contractile process. Dynamic activation and shortening are required for the muscle to perform work and therefore during contraction and relaxation the muscle will undergo a range of different length changes. Similarly, a muscle cannot shorten indefinitely, suggesting that eventual re-lengthening will be required, therefore during *in vivo* contraction there will be a period where the muscle will be producing negative work (Josephson, 1993).

During isotonic contractions the muscles are shortened and lengthened while maintaining a constant force. Hence, the amount of work done on the muscle to

lengthen is the same as the amount of work done by the muscle to shorten. Muscle properties are evaluated by exploring the relationship between the muscle force and velocity of shortening. Furthermore, the combination of force and length changes are unrealistic as shortening velocities and force production vary greatly during cyclical muscle movement. Unlike contractiling ventricles, during isometric and isotonic contraction studies no mechanical work is performed. Moreover, it has been shown that isometric and isotonic contraction studies overestimate the amount of work undertaken by the muscle during contraction and relaxation in both skeletal and cardiac muscle (James et al., 1996, Mellors et al., 2001).

5. Work-loop contraction studies

It has been demonstrated that isometric and isotonic contraction studies poorly estimate the true *in vivo* muscle function (James et al., 1996, Mellors et al., 2001). Research by Josephson et al., (1985) developed the *in vitro work-loop* technique which resembled *in vivo* cardiac biomechanical motion more closely, as it considers multiple factors that determine muscle performance such as pre-stretch effects, activation events and shortening dependent deactivation (Josephson, 1985). Muscles usually work in antagonistic pairs thereby going through cyclical length changes during contraction and relaxation.

The work-loop technique applies sinusoidal length changes to estimate *in vivo* muscle function. It has been suggested that length changes that occur in the *in vivo* settings are not exactly in sinusoidal shape and form but are much more

complex in nature. However, the sinusoidal muscle has been considered to be a good and easy to implement generalisation of the *in vivo* muscle mechanics (Mellors et al., 2001, Mellors and Barclay, 2001).

Ventricular contraction and relaxation can be separated into three phases in relation to the variation in muscle strain patterns; an initial isometric phase occurs during iso-volumic contraction at the start of ventricular systole, a shortening phase during ventricular ejection and finally re-lengthening phase that coincides with ventricular filling (Sperelakis, 2001). Thus sinusoidal length changes during muscle activation and relaxation represent a closer simulation of *in vivo* contractions. The work-loop technique can be used to combine sinusoidal length changes with phasic electrical stimulation, allowing power output of the muscles during contractions to be investigated under realistic physiological conditions (Josephson, 1985, Syme and Josephson, 1995, Josephson, 1993, James et al., 1996, Caiozzo, 2002).

One of the key advantages of the work-loop technique is that it considers both passive and active properties of the muscle during contractions. In the work-loop the muscles are stimulated during lengthening, the muscles will continue to lengthen until the muscles produce maximum force. During lengthening the muscles are not producing work; in contrast work is required for this phase of muscle contractions. Therefore, the net-work required for the lengthening phase is negative (Josephson, 1985, James et al., 1996. Following the rapid activation phase, the muscles undergo shortening, during which they produce work. Net-work during shortening phase is positive work (James et al., 1996, Josephson,

1993). Parameters that can affect power output during sinusoidal contraction include cycle frequency used, the strain (% length change) of the muscle and the electrical stimulation phase and pattern (Josephson, 1993).

By plotting the force produced by the muscle against muscle length, work and power output of the muscle can be calculated. Unlike the isometric or isotonic contraction model, the work-loop technique also takes into account the work done by the muscle during shortening as well as the work required to re-extend the muscle during lengthening (Josephson, 1985, James et al., 1996). This technique also incorporates changes in shortening velocity and the level of muscle activation during contractions as well as producing force and length changes that closely resemble papillary muscle dynamics *in vivo* (Semafuko and Bowie, 1975, Hirakawa et al., 1977). Thus it is clear that the work-loop technique provides a more realistic physiological representation of cardiac muscle mechanics, allowing for a more accurate assessment of pharmacological compounds on cardiac muscle performance (Layland et al., 1995, Layland and Kentish, 1999, Layland and Kentish, 2000). Studies from our laboratory have shown detrimental effects of doxorubicin on papillary muscle contraction using the work-loop technique. We showed that clinically relevant concentration of doxorubicin caused a time-dependent decrease in muscle power output, maximum force and changes in the work-loop shapes (Gharanei et al., 2014).

Therefore, the work-loop is considered to be an ideal choice for investigating muscle mechanics in health, stressed and diseased states, in particular when working with cardiac muscle.

6. Cellular Impedance

As discussed above, various cardiac preparations ranging from papillary muscle to Langendorff preparations have been used to investigate drug effects on cardiac inotropy. In addition to these preparations, primary cardiomyocyte preparations have also been utilized to assess sarcomere length changes as a screen to assess contractility (Harmer et al, 2012). Technical difficulties in obtaining sufficiently pure high yield cardiomyocyte preparations (Harding, Ali, Brito-Martins & Gorelik 2007) and the limited through-put of these methods often prevents their use for screening purposes during early drug development.

Recently the impedance signals from various cells including neonatal rat myocytes and stem cell derived cardiomyocytes such as induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM) and mouse embryonic stem cell derived cardiomyocytes have been investigated as surrogate markers for contractility (Scott et al, 2014). It is beyond the remit of this review to discuss the current status of stem cell derived cardiomyocytes in pharmacology screening please refer to Khan et al (2013) and Kraushaar et al (2012) for a detailed review of iPSC-CM in pharmacological screening. Needless to say although iPSC-CM have been demonstrated to exhibit molecular and fundamental properties of the human heart (Himmel et al., 2013) detailed analysis has revealed a mixed fetal/adult expression pattern in general (Guo et al., 2011) and in particular immature expression of key proteins associated with calcium handling (Lee et al., 2011).

Overall results have been varied and responses from the stem cell derived cardiomyocytes have been shown to respond, on occasion, differently from those originating from established *in-vitro* and *in-vivo* models (Himmel 2013). The assay also has inherent limitations with the inability to directly measure the velocity of contraction (Doherty et al 2013). Although it has been shown that drug-induced cardiotoxic effects may be detected within this system, with the potential to identify cardiotoxicity hazard suitable for use in high throughput screening, this technique is an indirect measure of contractility and has provided contradicting results. Cellular impedance does not provide insight into the intricate mechanisms implicated in contractility related cardiotoxicity and at this present time has not been demonstrated to be a useful tool to predict risk identification. Overall the translational value of results from the cellular impedance assay is considered limited and not yet firmly established (Himmel 2013).

7. Literature review of the concordance between drug effects on cardiac contractility in man and *in vitro* assays of cardiac contractility.

As discussed above there are a number of *in vitro* assays that have been used to evaluate the potential effects of compounds on cardiac contractility. However, we are unaware of any evaluation of the predictivity of these assays for effects in humans. In order to address this knowledge gap we have conducted a literature review on 22 compounds to understand the concordance between the non-clinical assays and human outcome. The human data was based on studies that measured drug effects on cardiac contractility, usually by echocardiography or cardiac catheterization. The one exception to this was itraconazole in which the

clinical findings were based on clinical reports of symptoms of heart failure and the introduction of the FDA black box warning. Some clinical studies measured the drug plasma concentrations within the cardiac contractility study whilst for other compounds the drug levels were estimated from published pharmacokinetic data. The free drug levels were estimated using published human plasma protein binding data often found in the drugbank.ca website. The non-clinical assessment was based solely on *in* vitro data.

The literature search revealed the use of a wide range of assay types ranging from perfused whole hearts (Langendorff preparations), to isolated papillary muscle, ventricles and atria. In addition, hearts were removed from a wide range of species, including rat, guinea pig, rabbit and cat, with the guinea pig being the most common species. The heterogeneous nature of the dataset created several challenges with respect to defining the relationship between the *in vitro* assays and findings in man. Firstly, there is no agreed standard for the definition of the magnitude of change that constitutes a drug effect in both the non-clinical assays and also effects in man. Therefore we reported the author's definition of 'a concentration effect'. Secondly, there is no 'standardised' protocol for the conduct of the non-clinical assays making comparisons across studies challenging and therefore again we have reported the data as per the study report with no further analysis. Thirdly, as previously mentioned the issue of species variation. There was insufficient data to analyse individual species with respect to the concordance with man and so we have conducted the analysis independent of species and asked the question, does the non-clinical assays published in the literature predict effects in humans? Despite these challenges

we have attempted to address two critical questions. How reliable are the available assays at identifying potential hazards with respect to effects on cardiac contractility in man and secondly can the available assays be used to manage a cardiac contractility risk? As a hazard identification tool, safety scientists will use an assay to identify a potential drug hazard (in this case an effect on cardiac contractility) at any concentrations regardless of the relevance to the human exposure. Thus, this question can be addressed by assessing the concordance between the clinical data and an *in vitro* cardiac contractility signal at any concentration in the latter. Once a hazard has been identified it can be managed through a number of actions. Firstly an understanding of the potential safety margin for the effect when compared to the predicted human exposure, secondly by understanding the mechanism of action (which should help define the human relevance) and thirdly by conducting additional studies, including an *in vivo* evaluation. We have attempted to assess the value of the available *in vitro* cardiac contractility assays as risk management tools by assessing the concordance between the clinical data and the *in vitro* non-clinical data at a set multiple (~10-fold) of the clinical exposure. Thus addressing the ability of the current assays to predict the likely human exposure that may cause an effect on cardiac contractility (i.e. ability to be used to predict the likely safety margin in man). One observation from the review is that a number of drugs had a range of different potencies (>10-fold) across the various tissue types (e.g. amrinone, dobutamine, phenylephrine, cibenzoline and verapamil) and to a lesser extent on the same assay/species (e.g. disopyramide). Thus, to assess the value as a risk management tool, we have taken a conservative approach and compared the most potent value in the non-clinical assay with the clinical findings.

The available assays appear to have good concordance for humans with respect to hazard identification. Thus, the assays correctly identified, at some concentration, a range of human positive inotropes (including amrinone, digoxin, levosimendan, milrinone and enoximone) as well as negative inotropes (atenolol, flecainide, itraconazole and verapamil) and neutral compounds (captopril, metoprolol and phentolamine). The exceptions to this were clonidine (negative inotrope in man but a positive inotrope *in vitro*), sunitinib (negative inotrope in man, but inactive on the guinea pig Langendorff) and the calcium channel blockers nifedipine and diltiazem which have been shown to have no effect on cardiac contractility in man at therapeutic concentrations and yet have a negative inotropic effect on a range of cardiac preparations. The negative inotropic effect of clonidine in man is most likely due to it's known effects on sympathetic drive (Cavero and Roach 1980) and not a direct effect on the heart and thus such an effect would not be expected to be detected *in vitro.* Thus, it must be recognised that *in vitro* cardiac contractility assays will only detect direct drug effects on the heart and not those changes mediated through central/reflex mechanisms. The adverse effects of sunitinib on cardiac function in man can take many days to develop (>22 days) (Orphanos, Ioannidis and Ardavanis 2009) and may involve some remodeling of the heart. Thus drug effects on cardiac function that may be secondary to structural changes, as opposed to direct functional effects, may not be detectable in acute safety pharmacology studies. The negative inotropic effects of nifedipine and diltiazem *in vitro* that was not observed in humans may be due to the reflex increase in cardiac contractility in man secondary to vasodilation, which could overcome the

direct negative inotropic action of these drugs. Nevertheless, it is not surprising that some compounds will profile as 'false positives' in the non-clinical assay (an effect *in vitro* not observed in humans, in this case a negative inotropic effect).

The ability of an assay to correctly predict the clinical outcome is referred to as the true positive (TP) rate (in this analysis it is slightly complicated because a true positive is a compound with a positive or negative inotropic effect *in vitro* which translates to the same effect in humans), whereas the false positive (FP) compounds are those with positive or negative inotropic activity *in vitro* that do not translate to man, true negatives (TN) have no effect *in vitro* nor in man (neutral compounds) and false negative (FN) compounds have no effect *in vitro*, but have an effect on cardiac contractility in man. By comparing the true positive/negative and false positive/negative rate the assay sensitivity (TP/TP+FN) and specificity (TN/TN+FP) can be calculated. As a hazard identification tool (an effect at any concentration *in vitro*), the assays performed well with a total of 15 true positives (all positive inotropes and all negative inotropes except clonidine and sunitinib), 2 false negatives (clonidine and sunitinib), 3 true negatives (captopril, metoprolol and phentolamine) and 2 false positives (diltiazem and nifedipine) (table 2). These data would suggest that the sensitivity of the available non-clinical *in vitro* assays is good (88%), although the specificity relatively poor (60%). This may be related to the small number of true negatives in this dataset.

Although the *in vitro* assays identified all of the positive inotropes in man, there were some significant potency differences. For example, pimobendan has very

potent inotropic effects in man (0.0005 μM free drug levels, table 1) and yet is relatively weak *in vitro* (10 to 50 μM, table 1). Likewise, digoxin is a potent inotrope in man (0.001 μM) and much weaker *in vitro* (0.2 to 0.3 μM). Interestingly, levosimendan (0.013 μM) is less potent than pimobendan (0.0005 μM) in man, but more potent *in vitro* (table 1). Therefore it appears that the assays do not always predict the likely free drug levels that may affect cardiac contractility in humans. This is an important aspect of risk management. To assess the value as a risk management tool the concordance of the assays with man has been compared using the most potent non-clinical value with concentrations up to ~10-fold higher than those shown to have an effect on cardiac contractility in man. As an example, amrinone has a positive inotropic effect in man following exposures to free drug levels of 15 μM and has a positive inotropic effect *in vitro* at 107 μM and is therefore considered a true positive (an effect *in vitro* at <150 μM, 10 x 15 μM). In contrast pimobendan would be considered a false negative since there was no effect *in vitro* at concentrations up to 0.005 μM (10 x 0.0005 μM). Using this type of analysis two compounds emerge as 'threshold', phenylephrine and enoximone, since these positive inotropes have effects *in vitro* at 20-fold and 13-fold the clinical concentrations. Thus the data has been reported with these compounds as both true positive and false negatives.

Based on this analysis 12 true positives were identified (amrinone, dobutamine, levosimendan, milrinone, phenylephrine, enoximone, atenolol, cibenzoline, disopyramide, flecainide, itraconazole and verapamil), 5 false negatives (digoxin, glibenclamide, pimobendan, clonidine and sunitinib), 3 true negatives (captopril,

metoprolol and phentolamine) and 2 false positives (diltiazem and nifedipine) (table 3). These data would suggest that, as a risk management tool, the sensitivity of the available non-clinical *in vitro* assays is lower (70%) than that as a hazard identification tool, although the specificity remains the same (60%). If phenylephrine and enoximone were classified as false negatives the assay sensitivity would fall to 58%. One potential criticism of using the most potent *in vitro* potency to compare with human data is that this may increase the false positive rate (i.e. an effect *in vitro* not observed in man), however this does not appear to be the case because only 2 false positive compounds were identified (diltiazem and nifedipine) and the potency estimates were quite consistent across the literature.

In conclusion this literature review has identified a wide range of different *in vitro* assays, taken from different species, to assess drug effects on cardiac contractility. The available assays appear to perform well as a hazard identification tool (sensitivity = 88%, specificity = 60%), but not so well at predicting the likely concentrations that may affect cardiac contractility in man (sensitivity = 58 - 70%, specificity = 60%). Furthermore, the non-clinical assays did not effectively rank order the potency of compounds with the same mode of action (pimobendan and levosimendan). Thus there appears to be a need for new and improved cardiac contractility assays that may better predict the concentrations that may affect cardiac contractility in man. The data would also suggest that the available *in vitro* assays could be incorporated into an early safety testing strategy as hazard identification tools with the risk management of

any findings being dependent on the use of new assays e.g. *in vitro* work-loop assay and/or *in vivo* assessment.

Table 1. Summary of literature search with inotropes of known clinical outcome and corresponding non-clinical *in vitro* data.

Compound	Human Data	Estimated drug levels	Non-clinical finding
Positive inotropes			
Amrinone	600 mg daily to heart failure patients.[1] Intravenous dosing (0.5 to 3.5 mg/kg) to heart failure patients.[2] Inotropy measured by right heart catheterization.	3.7 μg/ml[2] = 15 μM free.	Guinea pig Langendorff (107 μM)[3], rabbit papillary muscle (1000 μM)[4], cat papillary muscle (300 and 1000 μM).[5,6]
Digoxin	20 μg/kg orally to healthy volunteers.[7] Changes in the pre-ejection period index (PEPI) obtained from the systolic time intervals used as a measure of inotropism.	0.5 to 1.0 ng/ml[7] = 0.001 μM free.	Guinea pig papillary muscle (300 nM)[8] and cat papillary muscle (200 nM).[9]
Dobutamine	20 μg/min infused into left coronary artery of healthy volunteers and heart failure patients.[10] Inotropism measured following left ventricular catheterization.	200 ng/ml[10] = 0.5 μM free.	Papillary muscle: cat (0.1 μM)[11], guinea pig (2 μM)[6], cat (2 μM)[6]. Guinea pig atria 2.6 μM[12] and 0.5 μM.[6]
Glibenclamide	5 mg glyburide to healthy volunteers.[13] Inotropism measured radionucleotide ventriculography.	637.9 nmol/l total[14] = 0.06 μM free.	Rabbit Langendorff (10 μM).[15]
Levosimendan	3 mg levosimendan to healthy volunteers. Effects measured by M-mode echocardiography.[16]	180 ng/ml[17] = 0.013 μM free.	Papillary muscle: rabbit (0.1 μM)[18] and guinea pig (0.1 μM)[19]. Guinea pig atria (0.1 μM).[19]
Milrinone	0.5 μg/kg/min to heart failure patients. Inotropy measured by right heart catheterization.[20]	352.3 ng/ml[20]= 0.3 μM free.	Langendorff: guinea pig (1.0 μM)[3], rabbit (7.7 μM)[21]. Guinea pig papillary muscle (8.0 μM).[22]
Phenylephrine	20-200 μg /min infused into left coronary artery of healthy volunteers and	Intracoronary concentration of 1.0 μM	Papillary muscle: guinea pig (10 μM)[23], cat (1.0 μM)[6]. Rat atria (10 μM).[24]

	heart failure patients.[10] Inotropism measured following left ventricular catheterization.	total[10] = 0.05 µM free.	
Pimobendan	7.5 mg p.o. or 5 mg i.v. in healthy volunteers. Inotropy measured by echocardiography.[25]	PKPD modeling suggests an effect concentration of 6.5 ng/ml[25] = 0.0005 µM free.	Papillary muscle: guinea pig (21 µM)[22], (50 µM)[26], (10 µM).[27]
Enoximone	1.5 mg/kg i.v. to patients with coronary artery disease and left ventricular dysfunction. Inotropy measured by echocardiography.[28]	Total concentrations of 5 nmol/ml[29] = 0.75 µM free.	Guinea pig Langendorff (10.0 µM).[3]
Negative inotropes			
Atenolol	48 weeks of treatment with atenolol or irbesartan in patients with hypertensive LV hypertrophy. Primary end point was Doppler-derived myocardial performance index.[30]	Total therapeutic concentrations of 550 ng/ml[31] = 0.2 µM free.	Rat left and rat atria (1 µM).[32]
Cibenzoline	Initial oral dose of 260 mg followed by 130mg b.i.d. in patients with nonsustained ventricular tachycardia and left ventricular dysfunction. Inotropy measured by right heart catheterization.[33]	Total peak plasma concentrations of 1362 ng/ml[33] = 2.5 µM free.	Guinea pig papillary (30 µM)[34], (35 µM)[35], rabbit atria (1.32 to 5.26 µM).[36]
Clonidine	1 µg/kg i.v. in healthy subjects. Cardiac function measured by impedance cardiography.[37]Decreased Inotropy in heart failure subjects measured by isovolumic contraction.[38]	Therapeutic plasma concentrations 1.3 ng/ml[39] = 0.004 µM free.	Guinea pig papillary – **increased** force contraction (5.0 µM).[23]
Disopyramide	1.5 mg/kg i.v. to healthy volunteers. Cardiac contractility measured by echocardiography.[40]	Peak plasma concentrations of 6.8 µg/ml[40] = 9.0 µM free.	Guinea pig papillary (10 µM)[41] and (100 µM).[42]
Flecainide	800 mg orally to healthy	Peak plasma	Guinea pig papillary muscle

	volunteers, cardiac contractility measured by echocardiography.[43]	concentrations of 296 ng/ml[43] = 0.4 μM free.	(5 μM)[42], rat and rabbit ventricle (10 μM).[44]
Itraconazole	Clinical signs of heart failure.[45]	Therapeutic plasma concentrations ~2 μg/ml[46] = 0.03 μM free.	Rabbit Langendorff (0.3 μM).[47]
Sunitinib	Reductions in left ventricular ejection fraction inpatients with metastatic renal cell carcinoma.[48]	Therapeutic plasma concentrations ~100 ng/ml[49] = 0.01 μM free.	No effect on guinea pig Langendorff at 0.3 μM.[50]
Verapamil	10 mg i.v. in patents with coronary artery disease and rheumatic valvular disease. Inotropy measured by heart catheterization.[51]	Plasma concentrations of 40 ng/ml following 10 mg i.v. [52] = 0.01 μM free.	Guinea pig Langendorff (0.03 μM)[50]; Papillary muscle, rat (0.5 μM)[53] and guinea pig (0.8, 0.5 μM).[34,54]
Neutral compounds			
Captopril	50 mg b.i.d. in heart failure patients, cardiac contractility measured by echocardiography.[55]	Free drug levels of 300 ng/ml = 1.06 μM free.[56]	Langendorff - no effect in guinea pig (1.0 μM)[57] or rat (0.4 μM).[58]
Diltiazem	120 mg p.o. in coronary infarction patients. Inotropy measured by heart catheterization.[59]	Therapeutic plasma concentrations ~130 ng/ml[46] = 0.06 μM free.	Langendorff – **decreased** contractility in rat (1.0 μM)[60]and rabbit (1.0 μM)[61]. Reduced contractility in guinea pig papillary muscle (3.0 μM).[54]
Metoprolol	50 mg b.i.d. to healthy subjects, cardiac contractility measured by echocardiography.[62]	Total drug levels of 500 nM following 100 mg b.i.d. = 0.2 μM free following 50 mg b.i.d.[63]	No effect on guinea pig Langendorff (1.0 μM).[64]
Nifedipine	20 mg p.o. in coronary infarction patients. Inotropy measured by heart catheterization.[59]	Therapeutic plasma concentrations ~100 ng/ml[46] = 0.01 μM free	**Decrease** force - guinea pig papillary muscle (0.1 μM).[54]
Phentolamine	200 μg /min infused into left coronary artery of healthy volunteers and	Intracoronary concentration of 5.0 μM	No effect on rat and rabbit papillary muscle (1.0 μM).[65,66]

	heart failure patients.[10] Inotropism measured following left ventricular catheterization.	total[10] = 2.25 µM free.	

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Table 2 2x2 Contingency table for the concordance of *in vitro* cardiac contractility assays with human findings regardless of concentration.

Non-clinical outcome		Clinical Outcome	
		Inotropic effect	No inotropic effect
	No inotropic effect	3	2
	Inotropic effect	2	15

Table 3 2x2 Contingency table for the concordance of *in vitro* cardiac contractility assays with human findings by comparing *in vitro* concentrations up to 10-fold higher than those tested in man .

Non-clinical outcome		Clinical Outcome	
		Inotropic effect	No inotropic effect
	No inotropic effect	3	5
	Inotropic effect	2	12

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