The present invention relates to methods of screening test substances in order to evaluate their toxic or protective effects on mammalian cells. In particular, the invention relates to the methods of obtaining an indication of the cardiotoxic or cardioprotective effect of a test substance by detecting or measuring the production of miRNA by mammalian cells which have been contacted with the test substance.
ASSAYS FOR SCREENING DRUG SAFETY

The present invention relates to methods of screening test substances in order to evaluate their toxic or protective effects on mammalian cells. In particular, the invention relates to the methods of obtaining an indication of the cardiotoxic or cardioprotective effect of a test substance by detecting or measuring the production of miRNA by mammalian cells which have been contacted with the test substance.

Testing of all drug candidates is required by regulatory authorities in order to establish the clinical safety of such drugs. One particular concern is cardiovascular toxicity. Such toxicity can result from excessive accumulation of toxic chemicals within heart tissue, cardiovascular-specific bioactivation of protoxicants and/or interference with specialised cellular functions. These effects can compromise the heart's pumping ability and hence the circulation of blood flow to major organs.

Doxorubicin and daunorubicin are anthracycline antibiotics and amongst the most successful of broad spectrum anticancer agents developed. They are used particularly in the treatment of solid tumours and haematological malignancies. Despite their importance in anticancer pharmacology, their clinical value is severely jeopardized by their cumulative and irreversible dose dependent cardiotoxicity. Anthracycline-induced cardiotoxicity can cause serious health problems for an increasing number of children surviving childhood malignancies (Saltzer et al., 2010 Leukemia 24:355-370) and early detection of cardiac failure is critically important for its prevention and management (Higgins et al. (1987) Lancet 2(8563):863-864). Both acute and chronic cardiomyopathy occur following anthracycline chemotherapy such as doxorubicin treatment (Belham, M., et al., (2007) European journal of heart failure, vol. 9, no. 4, pp. 409-414). Recent studies suggest an incidence of this type of cardiotoxicity of 5% in doses up to 400 mg/m² increasing to 48% in subjects receiving 700 mg/m² (Swain SM et al., 2003 Cancer 97:2869-2879). But even at doses up to 150 mg/m² CHF was occasionally reported (Swain SM et al., 2003 Cancer 97:2869-2879). In addition to the cumulative dose, age, gender and dosing schedule have been reported as independent risk factors (Von Hoff et al., 1979 Ann Intern Med 91:710-717).

Both in vitro and in vivo assays for testing new drug candidates are currently required by the FDA guidelines. Traditional in vitro assays are either on a molecular level (e.g. affinity binding assays, heterologous hERG channel expression), on a cellular level (e.g. hERG channel
block in native cardiac myocytes), on a tissue level (e.g. repolarisation assays on papillary muscle or Purkinje fibres) or on an organ level (e.g. Langendorff heart). Whilst the more complex systems provide a closer approximation to the patient, such complexity is associated with higher costs and less opportunity for high-throughput screening. Hence the current requirements of the regulatory authorities has lead to a bottleneck in the development of new drugs.

Heart disease is the most common cause of death in the elderly, and this has resulted in an increasing number of patients older than 70 years undergoing clinical treatment of myocardial infarct by thrombolytic therapy and revascularization by percutaneous, coronary intervention or coronary artery bypass graft surgery. Age dependence on effective cardioprotective therapies is highly relevant in a clinical setting, since the majority of patients are in the older age range: for example coronary artery disease affects 50% of those older than 65, with up to 80% of deaths from the disease occurring in this age group. With the continued aging of populations in coming decades, the proportion of people in this age range will grow considerably (1 to 4 in developed countries). There is an urgent need therefore for appropriately-tested drugs to treat such diseases, and particularly drugs for the treatment of older members of the population.

There are a variety of shortfalls with the current techniques which have been proposed to assess myocardial injury including sensitivity, specificity high costs and their availability (Minotti, G., et al., (2004) Pharmacological reviews, vol. 56, no. 2, pp. 185-229; Bryant J. et al., (2007) Health Technology Assessment 2007; Vol. 11: No. 27; van Dalen E.C. et al., (2009) Cochrane Review, pp 1-63). The gold standards to detect drug-induced cardiotoxicity such as anthracyclines are cardiac imaging techniques or myocardial biopsy. However, these methods have either the disadvantage that cardiotoxicity is detected late, namely when decline in left ventricular ejection fraction (LVEF) already has occurred (imaging techniques) or that it is highly invasive and based on the assumption that the damage is equally distributed over the myocardium (biopsy) (Broeyer et al., J Cancer Res Clin Oncol. 2008 September; 134(9): 961-968). It is generally acknowledged that anthracyclin-induced cardiotoxicity becomes evident after completion of the chemotherapy. Animal studies have shown that anthracyclin-induced apoptosis can occur already after a single dose (Bennink RJ, (2004). J Nucl Med 45:842-848; Kumar D, (2001) Antioxid Redox Signal 3:135-145; Arola OJ, (2000) Cancer Res 60:1789-1792). This is line with the finding in humans that even at low cumulative doses cardiotoxicity have been reported (Swain SM et al., 2003 Cancer 97:2869-2879). If this could be measured and confirmed in humans, it might be possible to detect anthracyclin-induced cardiotoxicity in an
early stage. Unfortunately, the assessment of apoptosis itself in humans is difficult, but it can be hypothesised that the cell loss in the heart may be detected indirectly.

In addition many cardiac biomarkers are difficult to detect in the early stages of myocardial damage/failure (van Dalen E.C. et al., (2009) Cochrane Review, pp 1-63). The 'gold standard' in the clinical setting is the measurement of Troponin I following cardiac injury (Bryant J. et al., (2007) Health Technology Assessment 2007; Vol. 11: No. 27). The recently identified biochemical biomarkers brain-type natriuretic peptide (BNP) and atrial natriuretic peptide (ANP) have provided some promise for the identification of further biomarkers of cardiac dysfunction (van Dalen E.C. et al., (2009) Cochrane Review, pp 1-63). But both of these hormones are also expressed in other tissues, limiting their utility as specific cardiac biomarkers. One huge problem with the use of cardiac troponins and natriuretic peptides as markers of cardiac safety is that they only identify cardiotoxicity after the toxicity has extensively been established and therefore have limited value in their ability to assess early sub-clinical myocardial injury. Many cardiac biomarkers are not specific to the heart and elevation is difficult to detect in the early stages of myocardial damage/failure (Nikolaev V. O. et al., (2010) Science 26 March:Vol. 327. no. 5973, pp. 1653 - 1657). In addition to this, little is known about the use of these biomarkers to identify subclinical myocardial injury associated with drug induced cardiotoxicity. Cardiac biomarker surrogates which can be objectively measured and used as indicators for certain biological states or responses to pharmacological treatments are of increasing importance in many areas of modern medicine particularly in relation to the assessment of drug induced myocardial injury.

Recent studies have identified a role for miRNAs in a variety of basic biological and pathological processes, including various human diseases (Alvarez-Garcia i. & Miska E.A., (2005) Development 132 4653-4662). miRNAs are a class of endogenous non-protein-coding RNAs, usually comprising about 22 nucleotides. They regulate gene expression via RNA-induced silencing complexes, targeting them to mRNAs where they inhibit translation or direct destructive cleavage. Each miRNA is estimated to influence expression of hundreds of target genes, thereby regulating key cellular processes including proliferation, differentiation and survival including pathological processes and apoptosis (Wang Z., et al., (2008) J. Mol. Med. 86, 771-783; Ji X. et al., Clin. Chem. 2009; 55:1944-1949). Three miRNAs (i.e. miR-1, miR-133, and miR-208) are known to be highly enriched in the heart (Thum T. et al., Circulation. 2007;1 16:258-267). Distinct miRNA expression profiles have also been shown to be significantly altered in heart disease (e.g. human heart failure, hypertrophy, acute myocardial infarction and arrhythmias) (Yang B. et al., (2007) Nature Medicine vol. 13, No. 4 April 486-491;

There is a need, therefore, for new cardiovascular models to determine - early in the discovery or development process - whether a compound has the susceptibility to cause cardiotoxicity before large scale investment occurs in clinical studies.

The invention therefore provides a miRNA-based in vitro assay for the cardiotoxicity or cardioprotective effect of a substance, such as a new drug candidate. Such a method is capable of being utilised in an automated high-throughput screening assay, thus enabling the screening of new drug substances at a higher rate than previously achievable. Furthermore, through the use of particular cell types, the assay can determine the potential cardiotoxic or cardioprotective effect of candidate drugs on specified patient populations.

In a first aspect, therefore, the invention provides an in vitro method of obtaining an indication of the cardiotoxic or cardioprotective effect of a test substance, comprising the steps:

(i) contacting mammalian cells with the test substance,

(ii) detecting and/or measuring the production of one or more miRNAs by the mammalian cells in order to produce a miRNA profile for that test substance, and

(iii) comparing the miRNA profile of that test substance with one or more miRNA profiles produced by substances which are known to be cardiotoxic or cardioprotective, in order to obtain an indication of the cardiotoxic or cardioprotective effect of the test substance.

In a further aspect, therefore, the invention provides an in vitro method of obtaining an indication of the cardiotoxic or cardioprotective effect of a test substance, comprising the steps:

(i) contacting mammalian cells with the test substance,

(ii) detecting and/or measuring the level of one or more miRNAs produced by the mammalian cells, wherein the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and/or miR-542-3p,

in order to produce a miRNA profile for that test substance, and

(iii) comparing the miRNA profile of the test substance with one or more miRNA profiles produced by substances under the same conditions which have been shown to be cardiotoxic or cardioprotective, in order to obtain an indication of the cardiotoxic or cardioprotective effect of the test substance.
Preferably, the background level of miRNA which is produced by the mammalian cells in
the absence of the test substance is subtracted from the detected or measured miRNA levels
before comparison step (iii).

The invention also provides an in vitro method of obtaining an indication of the
cardiotoxic or cardioprotective effect of a test substance, comprising the steps:

(i) contacting mammalian cells with the test substance,
(ii) detecting the production of one or more miRNAs by the mammalian cells,

wherein the production of one or more miRNAs which are known to be produced upon contact
of the mammalian cells with a cardiotoxic substance is indicative of the cardiotoxic effect of the
test substance, and

wherein the production of one or more miRNAs which are known to be produced upon contact
of the mammalian cells with a cardioprotective substance is indicative of the cardioprotective
effect of the test substance.

Preferably, the background level of miRNA which is produced by the mammalian cells in
the absence of test substance is subtracted from the detected miRNA levels before the
relevance of the miRNA produced is considered.

The invention also provides an in vitro method of obtaining an indication of the
cardiotoxic or cardioprotective effect of a test substance, comprising the steps:

(i) contacting mammalian cells with the test substance,
(ii) measuring the level of one or more miRNAs produced by the mammalian cells,

wherein the miRNAs are selected from the group consisting of miR-1 , miR-21 , miR-27a,
miR-133a, miR-133b, miR146a, miR-181 and/or miR-542-3p,

wherein an increase or decrease in the level of the miRNA produced by the mammalian cells
compared to the level of the same miRNA produced by control mammalian cells in the absence
of the test substance is indicative of the test substance having a cardiotoxic or cardioprotective
effect.

Preferably, the method additionally comprises the step of determining whether the effect
is a cardiotoxic effect or a cardioprotective effect.

The invention further provides an in vitro method of obtaining an indication of the
cardiotoxic or cardioprotective effect of a test substance, comprising the steps:

(i) contacting mammalian cells with the test substance,
(ii) detecting and/or measuring a change in the production of one or more miRNAs by the
mammalian cells compared to control mammalian cells which have not been contacted
with the test substance,
wherein an increase in the production of one or more miRNAs whose expression is known to be increased upon contact of the mammalian cells with a cardiotoxic substance is indicative of the cardiotoxic effect of the test substance,

wherein a decrease in the production of one or more miRNAs whose expression is known to be decreased upon contact of the mammalian cells with a cardiotoxic substance is indicative of the cardiotoxic effect of the test substance,

wherein an increase in the production of one or more miRNAs whose expression is known to be increased upon contact of the mammalian cells with a cardioprotective substance is indicative of the cardioprotective effect of the test substance, and

wherein a decrease in the production of one or more miRNAs whose expression is known to be decreased upon contact of the mammalian cells with a cardioprotective substance is indicative of the cardioprotective effect of the test substance.

The invention further provides an in vitro method of obtaining an indication of the cardiotoxic or cardioprotective effect of a test substance, comprising the steps:

(i) contacting mammalian cells with the test substance,
(ii) detecting and/or measuring a change in the production of one or more miRNAs by the mammalian cells compared to control mammalian cells which have not been contacted with the test substance under the same conditions,

wherein the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and/or miR-542-3p,

wherein an increase in the level of one or more of the miRNAs whose level has been shown to be increased upon contact of the mammalian cells with a cardiotoxic substance under the same conditions is indicative of a cardiotoxic effect of the test substance, and

wherein a decrease in the level of one or more of the miRNAs whose level has been shown to be decreased upon contact of the mammalian cells with a cardiotoxic substance under the same conditions is indicative of a cardiotoxic effect of the test substance, and

wherein an increase in the level of one or more of the miRNAs whose level has been shown to be increased upon contact of the mammalian cells with a cardioprotective substance under the same conditions is indicative of a cardioprotective effect of the test substance, and

wherein a decrease in the level of one or more of the miRNAs whose level has been shown to be decreased upon contact of the mammalian cells with a cardioprotective substance under the same conditions is indicative of a cardioprotective effect of the test substance.
Preferably, the background level of miRNA which is produced by the mammalian cells in the absence of test substance is subtracted from the detected miRNA levels before the relevance of the miRNA produced is considered. In some embodiments of the invention, an increase in the level of miR-1 is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-1 is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-21 is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-21 is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-27a is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-27a is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-1 33a is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-1 33a is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-1 33b is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-1 33b is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-1 46a is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-1 46a is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-181 is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-181 is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-542-3p is indicative of a cardiotoxic effect of the test substance.
In some embodiments of the invention, a decrease in the level of miR-542-3p is indicative of a cardiotoxic effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-1 is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-1 is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-21 is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-21 is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-27a is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-27a is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-33a is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-33a is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-33b is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-33b is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-1 46a is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-1 46a is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-181 is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-181 is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, an increase in the level of miR-542-3p is indicative of a cardioprotective effect of the test substance.

In some embodiments of the invention, a decrease in the level of miR-542-3p is indicative of a cardioprotective effect of the test substance.
In some embodiments of the invention, a decrease in the level of one or more of the following miRNAs relative to a control is indicative of a cardiotoxic effect of the test substance or myocardial injury, particularly subclinical cardiotoxic induced myocardial injury: miRNA-133a, miRNA-133b, miRNA-21, miRNA-181.

In some embodiments of the invention, an increase in the level of one or more of the following miRNAs relative to a control is indicative of a cardiotoxic effect of the test substance or myocardial injury, particularly subclinical cardiotoxic induced myocardial injury: miRNA-1, miRNA-27a, miRNA-1 46a, miRNA-542-3p.

The *in vitro* method of the invention is carried out *in vitro* or *ex vivo*, i.e. it is not carried out on the human or animal body.

As used herein, the term "obtaining an indication of" a cardiotoxic or cardioprotective effect means obtaining information of an increased likelihood or statistically-significant chance (where in the increase/decrease is significant) of having a cardiotoxic or cardioprotective effect.

As used herein, the term "cardioprotective effect" means the drug is capable of contributing to the preservation of the heart by reducing or preventing myocardial damage, reducing or preventing primary or secondary prevention of coronary heart disease, reducing or preventing damage during cardio-surgical procedures, and/or reducing or preventing thrombolysis in acute myocardial infarction. All adaptive and compensatory mechanisms that directly or indirectly contribute to myocardial preservation are classified as "cardioprotective".

In particular, an effect may be termed a "cardioprotective effect" if the apoptotic and/or necrotic damage to heart cells under conditions of normoxia, hypoxia, hypoxia/reoxygenation or simulated ischaemia/reperfusion with the drug/test substance is less than that seen under corresponding control conditions without the drug/test substance.

Similarly, the term "cardiotoxic effect" is defined herein as the occurrence of heart muscle damage either causing muscle injury or altering cardiac excitability. The term "cardiotoxic effect" also covers a weakening of the heart and/or reducing its effectiveness in pumping and/or circulating blood.

In particular, an effect may be termed a "cardiotoxic effect" if the apoptotic and/or necrotic damage to heart cells under conditions of normoxia, hypoxia, hypoxia/reoxygenation or simulated ischaemia/reperfusion with the drug/test substance is more than that seen under corresponding control conditions without the drug/test substance.

The substance to be tested may be any product or composition whose effect, particularly whose cardiotoxicity or cardioprotective effect, needs to be tested. It may be a pure product or entity, or it may be a mixture of products or entities. In some embodiments, the substance may,
for example, be a chemical entity, such as a medicinal or veterinary drug. In other embodiments, the substance may be a biological molecule or composition, for example, a protein, polypeptide, peptide, enzyme, antibody, or a nucleic acid such as an antisense mRNA or miRNA. In some preferred embodiments, the test substance is a medicament which can be used to treat pulmonary disease, e.g. a bronchodilator or a glucocorticoid steroid, or a chemotherapy agent. One or more test substances may be applied to the cells simultaneously, separately or sequentially.

In the in vitro method of the invention, step (i) comprises contacting mammalian cells with the test substance. The mammalian cells may be any type of mammalian cells whose expression of a miRNA of interest changes upon contact with a cardiotoxic or cardioprotective substance. The term "mammalian cells" refers preferably to mammalian heart or muscle cells. More preferably, the mammalian cells are myocytes, myoblasts or cardiomyocytes. In some preferred embodiments of the invention, the mammalian cells are selected from the group consisting of mammalian adult and neonatal myocytes, human-derived cardiomyocyte stem cells, cardiomyocyte cell lines, skeletal muscle, rat skeletal muscle myoblasts (e.g. L6), Giradi, H2C9 cells or C2C12 mouse C3H muscle myoblasts.

Isolated cardio-myocytes are important for studying cardiac mechanics because they are free from connective tissue and endothelium, which cause a significant modification of tissue viscoelastic and contractile properties. In addition, subcellular mechanisms can be assessed in greater detail in single cardiomyocytes compared with intact tissue preparations. Preferably, therefore, the mammalian cells are cardiomyocytes (ventricular and/or atrial), most preferably human cardiomyocytes.

The cells may be obtained from any mammal, including rat, mouse, primate and human cells. Preferably, the mammalian cells are human cells.

In general, the mammalian cells are provided as a culture of mammalian cells. However, in some embodiments, the mammalian cells may be provided in the form of a tissue sample, preferably a heart or muscle tissue sample, most preferably atrial appendage or ventricular biopsy tissue.

As patients age, they experience progressive and irreversible changes in the morphology, function, and biochemistry of the cardiovascular system. This cardiovascular aging has been suggested as a cause for the adverse clinical prognoses that occur when ischemia results from myocardial infarction, coronary angioplasty, and cardiac surgery. Experimental studies have demonstrated that aged myocardium is less able to tolerate ischemic injury.
Furthermore, senescent cells display phenotypic characteristics which are distinct from young cells and are associated with alterations in gene expression, protein processing and altered metabolic processes comparable to the modifications that occur during the pathogenesis of many heart diseases. However, the molecular mechanisms that regulate gene expression in the ischemic heart during aging remain incompletely understood.

The present invention may therefore be used to evaluate the cardiotoxic or cardioprotective effect of test substances on specific patient populations through the use of mammalian cells which are representative of those patient populations. Examples include the use of the following:

(a) 3-6 month old primary cardiomyocytes as being representative of young cells (young subjects);

(b) 12 -18 month old primary cardiomyocytes as being representative of aged or senescent cells (aged subjects);

(c) myocardial tissue from 3-6 month old animals being representative of young tissue (young subjects); or

(d) myocardial tissue from 12-18 month old animals being representative of aged myocardial tissue (aged subjects).

In the above, the animals are preferably rats, rabbits, guinea pigs, dogs, cats or horses. Human tissues may also be used.

The cells are provided or cultured under conditions which allow for the miRNA to be tested to be expressed upon contact with a toxic or protective substance. Suitable conditions are well known in the art.

A further preferred aspect of the invention relates to an in vitro method as referred to herein wherein the mammalian cells are cultured under conditions which are representative of specific pathological conditions. One example of a specific pathological condition is ischemia (such as that resulting from myocardial infarct, coronary angioplasty or cardiac surgery).

Culture conditions are which representative of ischaemic conditions include culturing in a hypoxic buffer (e.g. 12 mM KCl, 0.49 mM MgCl2, 0.9 mM CaCl2, 4 mM HEPES, 10 mM Deoxyglucose and 20 mM lactate) in a hypoxic chamber (e.g. 6 hours in a hypoxic chamber pre-heated at 37°C, wherein the air in the chamber has been removed by a suction pump and replaced the 5% CO2 balanced in argon).

Culture conditions are which representative of a myocardial infarct include the use of an isolated rat heart preparation which is subjected to local ischemia/reperfusion. For example, isolated rat hearts may be subjected to about 20 minutes of stabilisation, about 35 minutes of
ischaemia and about 120 minutes of reperfusion, wherein the test substances are administered at the onset of reperfusion. An indicator such as Evans blue may be used to visualise the area of damage.

In some embodiments, the mammalian cells are cultured under conditions of ischemia and/or reperfusion.

A further aspect of the invention relates to an in vitro method as referred to herein, wherein the cells are cultured in a medium comprising a second test substance, as herein defined. The second test substance may, for example, be a bronchodilator or chemotherapy agent, e.g. ipratropium, cisapride, doxorubicin, salbutamol, salmeterol or budesonide.

Step (i) of the in vitro method comprises contacting the mammalian cells with the test substance. This may be done by adding the test substance to the culture medium surrounding cultured cells, injecting the test substance into the tissue sample or in any other suitable manner.

Step (ii) of the in vitro method comprises detecting and/or measuring the production of one or more miRNAs by the mammalian cells. The detection and measurement steps may be carried out by any suitable means. For example, the miRNA may be isolated from the cells by standard methods known in the art. Isolation may, for example, be by phenol/chloroform-based techniques, guanidine-HCl-based techniques or using miRNeasy columns (Qiagen). The yield and quality of any miRNA-enriched fractions may be monitored by a suitable spectrophotometric technique.

General methods of detecting miRNAs expressed by mammalian cells are well known in the art. These include microarray analysis, Northern blotting and reverse-transcription/DNA sequencing. Preferably, the detection and/or measuring of the miRNAs is carried out by microarray analysis.

Information may be obtained from any source on miRNAs which are known or have been shown to be expressed by the cells upon contact with cardiotoxic or cardioprotective substances. For example, this information may be available from published data sheets, computer databases, internet sources or produced in control experiments which have previously been performed, or from control experiments which are run simultaneously, separately or sequentially with the method of the invention.

By following the teachings of this invention, the person of skill in the art will be able to determine the miRNA profiles of substances which are known to be cardiotoxic or cardioprotective and to test substances to see whether they are likely to have cardiotoxic or cardioprotective effects.
As used herein, the term "miRNA profile" means a miRNA expression pattern which is characteristic of one or more miRNAs which are produced by the mammalian cells upon contact with a control or test cardiotoxic or cardioprotective substance.

The conditions used for the production of the test substance miRNA profile should ideally be the same as those used to produce the miRNA profiles for the substances which are known to be cardiotoxic or cardioprotective.

Preferably, the miRNA profile is made up of information from 500-1000, 100-500, 50-100, 40-50, 30-40, 20-30, 10-20, 5-10 or 1-5 miRNAs.

Preferably, the miRNA or miRNAs to be detected or measured in any aspect of the invention include one or more miRNAs selected from the group consisting of miR-1, miR1-1, miR1-2, miR-1 6, miR-1 8b, miR-20a/b, miR-21, miR-23, miR-24, miR-26a/b, miR-27a/b, miR-29a/b, miR-30a/b/c, miR-92a/b, miR-99, miR-100, miR-101, miR-1 25a/b, miR-126, miR-1 26-3p, miR-133a, miR133a-1, miR-133a-2, miR-133b, miR-143, miR-145, miR-145, miR-150, miR-195, miR-199*, miR-208, miR-328 and let-7.

In some embodiments, the miRNA or miRNAs to be detected or measured are miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and/or miR-542-3p.

In some embodiments of the invention, the most preferred miRNAs are miR-1, miR-27a, miR-133a, miR-133b, and/or miR-542-3p.

In some embodiments of the invention, the miRNA is not miR-1. Further details of the preferred miRNAs of the invention are given below:

Table 1:

<table>
<thead>
<tr>
<th>SEQ ID No.</th>
<th>Name (Sanger)</th>
<th>Mature miRNA sequence</th>
<th>Mature sequence reference</th>
<th>Stem Loop Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEQ ID No: 1</td>
<td>miR-1</td>
<td>UGGAAUGUAAGAAGAUGUGUAU</td>
<td>MIMAT0003125</td>
<td>M0003489</td>
</tr>
<tr>
<td>SEQ ID No: 2</td>
<td>miR-21</td>
<td>UAGCUUAUCAGACUGAUUGUA</td>
<td>MIMAT0000076</td>
<td>M0000077</td>
</tr>
<tr>
<td>SEQ ID No: 3</td>
<td>miR-27a</td>
<td>UUCACAGUGGCUAGUCCGC</td>
<td>MIMAT0000084</td>
<td>M0000085</td>
</tr>
<tr>
<td>SEQ ID No: 4</td>
<td>miR-133a</td>
<td>UUUGGUCCCCUCUCAACAGCUG</td>
<td>MIMAT0000427</td>
<td>M0000450</td>
</tr>
<tr>
<td>SEQ ID No: 5</td>
<td>miR-133b</td>
<td>UUUGGUCCCCCUCAACAGCUG</td>
<td>MIMAT0000770</td>
<td>M0000822</td>
</tr>
<tr>
<td>SEQ ID No: 6</td>
<td>miR-146a</td>
<td>UGAGAUCAGUAUUCGAGGCUU</td>
<td>MIMAT0000449</td>
<td>M0000477</td>
</tr>
<tr>
<td>SEQ ID No: 7</td>
<td>miR-181a</td>
<td>AACAUUCAACGCUGCUGAGU</td>
<td>MIMAT0000256</td>
<td>M0000269</td>
</tr>
<tr>
<td>SEQ ID No: 8</td>
<td>miR-542-3p</td>
<td>UGUGACAGAUUGAUAACUGAAA</td>
<td>MIMAT0003389</td>
<td>M0003686</td>
</tr>
</tbody>
</table>
The sequences of the above miRNAs are preferred for all aspects of the invention. Whilst the preferred sequences given above are human sequences, the invention is not limited to human sequences or to human miRNA. In particular, the invention relates to the above miRNA from any source, preferably animal miRNA, most preferably cat, dog, cow, cattle, mouse, rat, fish (e.g. zebrafish), horse, primates (e.g. gorilla), xenopus, rabbit, guinea pig, pig, goat, sheep, nematode, hamster, boar, or platypus miRNA. Preferably, the miRNA is a mammalian miRNA, most preferably rat, mouse or human miRNA.

As used herein, the term miRNA encompasses all forms of detectable miRNA, including pri-miRNA, pre-miRNA, miRNA precursors, miRNA/miRNA * duplexes and miRNP.

Preferably, the selected miRNA or miRNAs are ones whose production is characteristic of the test substance or which are statistically correlated or associated with the test substance.

In some embodiments, the miRNAs of ones that are capable of regulating genes encoding ion channels and/or transmembrane transporters.

Following the teachings of this invention, the person of skill in the art will also be able to determine the miRNA expression levels of individual substances which are known to be cardiotoxic or cardioprotective. If the production of such miRNAs is correlated with a cardiotoxic or cardioprotective effect, then the invention may be worked merely by the detection of such miRNAs, i.e. without the direct need to make any comparison between the levels of miRNA produced by the test substance and that produced by a control substance.

The invention may also be worked by detecting and/or measuring changes in the production of one or more miRNAs compared to control mammalian cells which have not been contacted with the test substance.

A particular miRNA may be said to be detected when that miRNA has a measured value above or below a threshold value for that miRNA. The threshold value may be obtained by obtaining a measured value of the miRNA in a control biological sample obtained from a control subject that has no disease conditions and not previously subjected to the substance. Alternatively, the threshold value may be obtained by obtaining a measured value of the miRNA in a control biological sample obtained from a control subject that has a disease condition and has previously been subjected to the substance.

The measured level of miRNA may be compared to a threshold value. If the measured miRNA value is above the threshold value, then the subject is identified as being at risk of a cardiac condition. Alternatively, if the measured value is below the threshold value, then the subject is identified as not being at risk of a cardiac condition or the subject is identified as having an improved cardiac condition.
Preferably, the changes in the levels of the miRNAs are significant changes, i.e. increases or decreases of at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% compared to the corresponding levels of miRNAs produced by the control (non-contacted) mammalian cells.

A signal log ratio \( > \pm 0.5 \) (1.4 fold) and p value <0.05 may be chosen as being statistically significantly altered by test substance.

In the event that some of the miRNA levels of interest which are being measured increase and some decrease, the overall predictive value of the method may be obtained by taking a weighted average of the measured values or by any other suitable statistical technique.

Wilcoxon rank sum tests may be used to compare the expression of one or more miRNAs in one or more subjects. Wilcoxon signed rank tests may be used to compare miRNA levels at different time points. Univariable linear and/or logistic regression analyses may be used to evaluate the relationships between a particular miRNA and other clinically-related indexes (e.g. QRS, ST segment, cTnl, CKMB, and blood pressure). Age and sex may be considered as controlled variables. Receiver-operator characteristic (ROC) analyses may be performed with miRNA levels plotted against a clinically-related variable. Area under ROC curve (AUC) may be used to assess the predictive power.

When miRNA profiles are compared in order to obtain an indication of an effect or susceptibility, diagnosis, optimal treatment, prognosis, disease progression, exposure, etc., the miRNA profiles may be compared by standard techniques, in particular by one of the above-mentioned techniques. When the two miRNA profiles are statistically not dissimilar, then it is likely that the effect, susceptibility, diagnosis, optimal treatment, prognosis, disease progression or exposure of the subject having the first miRNA profile may be said to apply to the subject having the second profile.

In a further aspect of the invention, the \textit{in vitro} method additionally comprises the steps of identifying a test substance having a cardioprotective effect and producing a pharmaceutical composition comprising that test substance, optionally together with one or more pharmaceutically-acceptable excipients, carriers and/or diluents. The invention further provides test substances having a cardioprotective effect which have been identified as a result of the method disclosed herein.

In yet a further aspect of the invention, there is provided a method of obtaining an indication of the therapeutic or adverse effect of a test substance which has been administered to a subject, the method comprising the steps:
(i) detecting and/or measuring the levels or amounts of one or more miRNAs produced in a first biological sample obtained from the subject in order to produce a miRNA profile of that first biological sample;

(ii) comparing the miRNA profile of that first biological sample with one or more miRNA profiles produced in second biological samples obtained from one or more subjects who are known to have been administered with therapeutic or adverse substances, in order to obtain an indication of the therapeutic or adverse effect of the test substance.

Preferably, the adverse effect is a toxic or cardiotoxic effect. Preferably, the therapeutic effect is a cardioprotective effect.

Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.

The invention further provides a method of obtaining an indication of the therapeutic or adverse effect of a test substance which has been administered to a subject, the method comprising the step:

(i) detecting the production of one or more miRNAs which are produced in a biological sample obtained from the subject, wherein the production of one or more miRNAs which are known to be produced in biological samples from subjects to whom a therapeutic substance has been administered is indicative of a therapeutic effect of the test substance, and

wherein the production of one or more miRNAs which are known to be produced in biological samples from subjects to whom a substance having a known adverse effect has been administered is indicative of an adverse effect of the test substance.

The invention further provides a method of obtaining an indication of the therapeutic or adverse effect of a test substance which has been administered to a subject, the method comprising the step:

(i) detecting the production or level of one or more miRNAs which are produced in a biological sample obtained from the subject, wherein the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p,

wherein the production of one or more of miRNAs which have been shown to be produced in biological samples from subjects to whom a therapeutic substance has been administered is indicative of a therapeutic effect of the test substance, and
wherein the production of one or more miRNAs which have been shown to be produced in biological samples from subjects to whom a substance having a known adverse effect has been administered is indicative of an adverse effect of the test substance.

The invention further provides a method of obtaining an indication of the therapeutic or adverse effect of a test substance which has been administered to a subject, the method comprising the steps:

(i) detecting and/or measuring a change in the production of one or more miRNAs produced in a first biological sample obtained from the subject compared to:

(a) one or more miRNAs produced in a biological sample obtained from a control subject, or

(b) one or more miRNAs produced in a second biological sample obtained from the subject before administration of the test substance,

wherein an increase in the production of one or more miRNAs whose expression is known to be increased upon administration of a subject with a therapeutic substance is indicative of the therapeutic effect of the test substance,

wherein a decrease in the production of one or more miRNAs whose expression is known to be decreased upon administration of a subject with a therapeutic substance is indicative of the therapeutic effect of the test substance,

wherein an increase in the production of one or more miRNAs whose expression is known to be increased upon administration of a subject with an adverse substance is indicative of the adverse effect of the test substance, and

wherein a decrease in the production of one or more miRNAs whose expression is known to be decreased upon administration of a subject with an adverse substance is indicative of the adverse effect of the test substance.

Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.

In this context, a control subject is preferably one to whom the test compound has not been administered. Preferably, the control subject is sex- and age-matched with the subject. In some embodiments of the invention, the adverse effect is a cardiac disease or disorder.

In a further embodiment, the invention provides a method of obtaining an indication of the susceptibility of a subject to a cardiac disease or disorder, the method comprising the steps:

(i) detecting and/or measuring the levels or amounts of one or more miRNAs produced in a first biological sample obtained from the subject in order to produce a miRNA profile of that first biological sample;
(ii) comparing the miRNA profile of that first biological sample with one or more miRNA profiles produced in second biological samples obtained from one or more subjects which are

(a) known to be susceptible to a cardiac disease or disorder, or

(b) known to be not susceptible to a cardiac disease or disorder,

in order to obtain an indication of the susceptibility of a subject to a cardiac disease or disorder. Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.

In yet a further embodiment, the invention provides a method of obtaining an indication of the susceptibility of a subject to a cardiac disease or disorder, the method comprising the steps:

(i) detecting the production of one or more miRNAs which are produced in a biological sample obtained from the subject, wherein the production of one or more miRNAs which are known to be produced in biological samples from subjects which are known to be susceptible to a cardiac disease or disorder is indicative of the susceptibility of the subject to a cardiac disease or disorder. Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.

In yet a further embodiment, the invention provides a method of obtaining an indication of the susceptibility of a subject to a cardiac disease or disorder, the method comprising the steps:

(i) detecting and/or measuring a change in the production of one or more miRNAs produced in a first biological sample obtained from the subject compared to one or more miRNAs produced in a biological sample obtained from a control subject who is known to be not susceptible to a cardiac disease or disorder, wherein an increase in the production of one or more miRNAs whose expression is known to be increased in subjects who are known to be susceptible to a cardiac disease or disorder is indicative of the susceptibility of the subject to a cardiac disease or disorder. Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.

Hence the invention provides a useful early indication of the susceptibility of a subject to one or more cardiac diseases or disorders. Such an early indication may be useful during standard health-screening of a patient.
It is particularly important for surgeons to be aware of the susceptibility of subjects to cardiac diseases or disorders prior to any surgical procedure. Hence, in one embodiment, the method is preferably carried out prior to a surgical procedure, e.g. as part of a pre-operative assessment of the subject. The surgical procedure may, for example, be angioplasty, coronary bypass graft (CABG) or mitral valve replacement.

In yet a further embodiment, the invention provides a method for obtaining an indication of the optimal treatment for a subject having a cardiac disease or disorder, the method comprising the steps:

(i) detecting and/or measuring the levels or amounts of one or more miRNAs produced in a first biological sample obtained from the subject in order to produce a miRNA profile of that first biological sample;
(ii) comparing the miRNA profile of that first biological sample with one or more miRNA profiles produced in one or more second biological samples obtained from one or more subjects prior to their successful treatment for a cardiac disease or disorder,

in order to obtain an indication of the optimal treatment for the subject having a cardiac disease or disorder.

Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.

In yet a further embodiment, the invention provides a method for obtaining an indication of the optimal treatment for a subject having a cardiac disease or disorder, the method comprising the steps:

(i) detecting the production of one or more miRNAs which are produced in a biological sample obtained from the subject,

wherein the production of one or more miRNAs which are known to be produced in biological samples from subjects which were subsequently successfully treated for a cardiac disease or disorder is indicative of that treatment for the subject having a cardiac disease or disorder.

Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.

In yet a further embodiment, the invention provides a method for obtaining an indication of the optimal treatment for a subject having a cardiac disease or disorder, the method comprising the steps:

(i) detecting and/or measuring a change in the production of one or more miRNAs produced in a first biological sample obtained from the subject compared to one or more miRNAs produced in a biological sample obtained from a control subject,
wherein an increase in the production of one or more miRNAs whose expression is known to be increased in biological samples from subjects who were subsequently successfully treated for a cardiac disease or disorder is indicative of that treatment for the subject having a cardiac disease or disorder, and

wherein a decrease in the production of one or more miRNAs whose expression is known to be decreased in biological samples from subjects who were subsequently successfully treated for a cardiac disease or disorder is indicative of that treatment for the subject having a cardiac disease or disorder.

Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.

In this context, a control subject is preferably one not having a cardiac disease or disorder. Preferably, the control subject is sex- and age-matched with the subject.

In yet a further embodiment, the invention provides a method for obtaining an indication of the prognosis of a subject having a cardiac disease or disorder, the method comprising the steps:

(i) detecting and/or measuring the levels or amounts of one or more miRNAs produced in a first biological sample obtained from the subject in order to produce a miRNA profile of that first biological sample;

(ii) comparing the miRNA profile of that first biological sample with one or more miRNA profiles produced in one or more second biological samples obtained from one or more subjects previously having a cardiac disease or disorder, in order to obtain an indication of prognosis for the subject having a cardiac disease or disorder.

Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.

In yet a further embodiment, the invention provides a method for obtaining an indication of the prognosis of a subject having a cardiac disease or disorder, the method comprising the steps:

(i) detecting the production of one or more miRNAs which are produced in a biological sample obtained from the subject,

wherein the production of one or more miRNAs which are known to be produced in biological samples from subjects previously having a cardiac disease or disorder is indicative of a decline in the prognosis of the subject having a cardiac disease or disorder.

Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.
In yet a further embodiment, the invention provides a method for obtaining an indication of the prognosis of a subject having a cardiac disease or disorder, the method comprising the steps:

(i) detecting and/or measuring a change in the production of one or more miRNAs produced in a biological sample obtained from the subject compared to one or more miRNAs produced in a biological sample obtained from a control subject, wherein an increase in the production of one or more miRNAs whose expression is known to be increased in biological samples from subjects having a more-advanced form of that cardiac disease or disorder is indicative of a detrimental progression or outcome of the disease or disorder in that subject.

Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.

In this context, a control subject is preferably one not having a cardiac disease or disorder. Preferably, the control subject is sex- and age-matched with the subject.

In yet a further embodiment, the invention provides a method for obtaining an indication of the exposure of a subject to a toxic substance, the method comprising the steps:

(i) detecting and/or measuring the levels or amounts of one or more miRNAs produced in a first biological sample obtained from the subject in order to produce a miRNA profile of that first biological sample;

(ii) comparing the miRNA profile of that first biological sample with one or more miRNA profiles produced in second biological samples obtained from one or more subjects who are known to have been exposed to one or more toxic substances, in order to obtain an indication of the exposure of the subject to a toxic substance.

In yet a further embodiment, the invention provides a method for obtaining an indication of the exposure of a subject to a toxic substance, the method comprising the steps:

(i) detecting the production of one or more miRNAs which are produced in a biological sample obtained from the subject, wherein the production of one or more miRNAs which are known to be produced in biological samples from subjects who are known to have been exposed to one or more toxic substances is indicative of the exposure of the subject to a toxic substance.

In yet a further embodiment, the invention provides a method for obtaining an indication of the exposure of a subject to a toxic substance, the method comprising the steps:

(i) detecting and/or measuring a change in the production of one or more miRNAs produced in a first biological sample obtained from the subject compared to:
(a) one or more miRNAs produced in a biological sample obtained from a control subject, or
(b) one or more miRNAs produced in a second biological sample obtained from
the subject before exposure of the subject to the toxic substance,

Wherein an increase in the production of one or more miRNAs whose expression is known to be
increased upon exposure of a subject to a toxic substance is indicative of exposure of the
subject to a toxic substance, and

Wherein a decrease in the production of one or more miRNAs whose expression is known to be
decreased upon exposure of a subject to a toxic substance is indicative of exposure of the
subject to a toxic substance.

In yet a further embodiment, the invention provides a method of screening for genes
involved in one or more cardiac diseases or disorders, comprising the steps:

(i) identifying one or more miRNAs which are overexpressed in subjects suffering from
one or more cardiac diseases or disorders compared to control subjects who are not
suffering from one or more cardiac diseases or disorders, and

(ii) identifying the target genes to which the miRNAs from step (i) bind,

Wherein the target genes to which the miRNAs from step (i) bind are ones which are
involved in one or more cardiac diseases or disorders.

As used herein, the term "cardiac disease or disorder" preferably refers to one or more
diseases or disorders selected from the group consisting of arrhythmia, heart failure, heart
disease, COPD/heart failure, myocardial infarction, coronary artery disease, acute coronary
syndrome, ischemic heart disease, cardiac hypertrophy, cardiomyopathy, aortic stenosis,
valvular dysfunction, diabetes, metabolic syndrome, vascular disease and heart surgery.

Preferably, the "cardiac disease or disorder" is arrhythmia. Examples of cardiac
arrhythmia include ventricular arrhythmia, a widening of the QRS complex and a prolonged QT
interval or a phase II arrhythmia.

As used herein, the term "biological sample" includes biopsy material, tissues,
mammalian cells, bodily fluids (e.g. whole blood, blood plasma, blood serum, cerebrospinal fluid,
saliva, seminal fluid, breast nipple aspirate, or urine) or combinations thereof. Preferably, the
biological sample is blood, blood plasma or blood serum.

The second biological sample or control biological sample may be obtained from the
subject prior to the subject being administered with or exposed to the substance. After
administration or exposure to the substance a biological sample is obtained and the measured
level of miRNA biomarker may be compared to the level in the control biological sample. For
example, the subject may be undergoing treatment with a substance that is known to cause, in some individuals, arrhythmias to the heart. In this example, the subject would be monitored before and after treatment with the substance by obtaining a biological sample and then detecting the presence or absence of a particular miRNA (e.g. miR-1) in that biological sample.

Preferably, the subject is a mammal, particularly preferably a human.

In some embodiments of the invention, the subject is a patient having a specific condition, e.g. a cardiac disease or disorder, or a cancer. The patients may be patients treated with a chemotherapy agent.

In some embodiments of the invention, in addition to the detection and/or measurement of the specified miRNA(s), one or more polypeptides may also be detected and/or measured. Such polypeptides may, for example, be serum response factor (SRF), proteins involved in hERG protein translation or proteins involved in potassium channel activity (e.g. Ikr).

In some embodiments of the invention, the indication or result is displayed as a written report that, optionally, provides a summary of the detected miRNA levels. In still other embodiments, the indication or result is outputted to a user interface device, a computer readable medium, or a local or remote computer system.

The invention further provides a kit comprising one or more pairs of primers for nucleic acid amplification and/or one or more probes for hybridization to a miRNA referred to herein; and instructional material for use of the primers and/or the probe to determine the presence or absence of the miRNA in a biological sample.

Alternatively, provided in the kit are one or more microarrays, e.g. oligonucleotide microarrays or cDNA microarrays, comprising probes that hybridize to the miRNA referred to herein and instructions for use of the microarray. In some embodiments the kits, comprising probes for miRNA biomarkers of cardiac condition, such as for example, one or more miRNAs selected from the group consisting of miR-1, miR1-1, miR1-2, miR-1-6, miR-1-8b, miR-20a/b, miR-21, miR-23, miR-24, miR-26a/b, miR-27a/b, miR-29a/b, miR-30a/b/c, miR-92a/b, miR-99b, miR-100, miR-101, miR-125a/b, miR-126, miR-126-3p, miR-133a, miR133a-1, miR-133a-2, miR-133b, miR-143, miR-145, miR-150, miR-195, miR-199*, miR-208, miR-328, and let-7; and, in suitable containers, control or reference samples to compare the patient / subjects measured miRNA values to; and instructions for use.

Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.

Most preferably, the miRNAs are selected from the group consisting of miR-1, miR-27a, miR-133a, miR-133b and miR-542-3p.
The invention also has uses in screening for test compounds which are useful in adjunct therapies. An adjunct therapy is an additional substance, treatment, or procedure used for increasing the efficacy or safety of the primary substance, treatment, or procedure or for facilitating its performance. The evaluation of interventions or treatments designed to prevent myocardial sub-clinical damage requires a robust early and preferably quantitative marker of the damage. Hence, there is a need for biomarkers that can be used to detect early drug-induced cardiotoxicity (e.g. chemotherapy agent anthracycline) (Broeyer et al., J Cancer Res Clin Oncol. 2008 September; 134(9): 961-968). There is also a need for compounds for use in such therapies.

In yet a further embodiment, therefore, the invention provides a method of screening for test compounds which are capable of reducing a cardiotoxic effect of a medicament, comprising the steps:

(i) contacting mammalian cells with a test substance and the medicament,
(ii) detecting and/or measuring a change in the production of one or more miRNAs by the mammalian cells compared to control mammalian cells which have been contacted under the same conditions with the medicament but not the test substance, wherein the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and/or miR-542-3p,

wherein a change in the level of one or more of the miRNAs is indicative that the test substance is capable of reducing or increasing a cardiotoxic effect of the medicament.

In yet a further embodiment, therefore, the invention provides a method of screening for test compounds which are capable of reducing a cardiotoxic effect of a medicament, comprising the steps:

(i) contacting mammalian cells with a test substance and the medicament,
(ii) detecting and/or measuring a change in the production of one or more miRNAs by the mammalian cells compared to control mammalian cells which have been contacted under the same conditions with the medicament but not the test substance, wherein the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and/or miR-542-3p,

wherein an increase in the level of one or more of the miRNAs whose level has been shown to be increased upon contact of the mammalian cells with a cardioprotective substance under the same conditions is indicative that the test substance is capable of reducing a cardiotoxic effect of the medicament, and
wherein a decrease in the level of one or more of the miRNAs whose level has been shown to be decreased upon contact of the mammalian cells with a cardioprotective substance under the same conditions is indicative that the test substance is capable of reducing a cardiotoxic effect of the medicament.

In yet a further embodiment, therefore, the invention provides a method of screening for test compounds which are capable of reducing a cardiotoxic effect of a medicament, comprising the steps:

(i) determining the level of one or more miRNAs which are produced by mammalian cells in the absence of the medicament,

(ii) determining the level of one or more miRNAs which are produced by mammalian cells in the presence of the medicament,

(iii) determining the level of one or more miRNAs which are produced by mammalian cells in the presence of the medicament and the test substance,

wherein steps (i), (ii) and (iii) are carried out under the same conditions,

wherein the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p,

wherein if the level of the miRNA which is determined in step (ii) increases compared to the level determined in step (i), then test substances which are found to reduce the level of miRNA which is produced in Step (iii) compared the level of miRNA produced in Step (ii) are test substances which are capable of reducing a cardiotoxic effect of the medicament, and wherein if the level of the miRNA which is determined in step (ii) decreases compared to the level determined in step (i), then test substances which are found to increase the level of miRNA which is produced in Step (iii) compared the level of miRNA produced in Step (ii) are test substances which are capable of reducing a cardiotoxic effect of the medicament.

The invention further relates to test substances which are obtained using the above method.

In some embodiments of the invention, the medicament is an anthracycline antibiotic, preferably doxorubicin or daunorubicin.

Cardiac damage after anthracycline therapy can be divided into early and late cardiotoxicity. Early cardiotoxicity refers to heart damage that develops during anthracycline therapy or in the first year after it is completed, and late cardiotoxicity refers to heart damage that only shows itself at least one year after the completion of anthracycline therapy (Bryant J. et
a/., (2007) Health Technology Assessment 2007; Vol. 11: No. 27). The risk of developing heart failure remains a lifelong threat, especially to children who have a long life-expectancy after successful treatment for cancer. Heart damage can occur as either subclinical cardiotoxicity or clinical cardiotoxicity. The term subclinical cardiotoxicity is used to describe various cardiac abnormalities, diagnosed with different diagnostic methods in patients without symptoms.

Clinical cardiotoxicity is defined on the basis of symptoms of clinical heart failure, confirmed by an abnormal diagnostic test. In the end stage of clinical heart failure, heart transplantation is the only remaining treatment option.

A further aspect of the invention therefore provides a method of obtaining an indication of the presence of subclinical myocardial injury in a subject, comprising the steps:

(i) detecting and/or measuring the levels or amounts of one or more miRNAs produced in a first biological sample obtained from the subject in order to produce a miRNA profile of that first biological sample;

(ii) comparing the miRNA profile of that first biological sample with one or more miRNA profiles produced from second biological samples obtained from one or more subjects which are

(a) known to have subclinical myocardial injury, or

(b) known not to have subclinical myocardial injury,

in order to obtain an indication of the presence of subclinical myocardial injury in the subject.

Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.

The determination of whether or not the on or more (other) subjects have subclinical myocardial injury may be based on echo-cardiography.

In yet a further embodiment, the invention provides a method of obtaining an indication of the presence of subclinical myocardial injury in a subject, comprising the steps:

(i) detecting the production of one or more miRNAs which are produced in a biological sample obtained from the subject,

wherein the production of one or more miRNAs which are known to be produced in biological samples from subjects which are known to have subclinical myocardial injury is indicative of the presence of subclinical myocardial injury in the subject.

Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.

In yet a further embodiment, the invention provides a method of obtaining an indication of the presence of subclinical myocardial injury in a subject, comprising the steps:
(i) detecting and/or measuring a change in the level of one or more miRNAs produced in a first biological sample obtained from the subject compared to the corresponding miRNA level in a biological sample obtained from a control subject who is known not to have subclinical myocardial injury, wherein an increase in the level of one or more of the miRNAs in the first sample from the subject compared to the control, which miRNA level has been shown to be increased in subjects which are known to have subclinical myocardial injury, is indicative of the presence of subclinical myocardial injury in the subject, and wherein a decrease in the level of one or more of the miRNAs in the first sample from the subject, which miRNA level has been shown to be decreased in subjects which are known to have subclinical myocardial injury, is indicative of the presence of subclinical myocardial injury in the subject.

Preferably, the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p.

The invention further provides the use of a miRNA selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p, in an in vitro method of diagnosing subclinical myocardial injury in a patient.

**20 BRIEF DESCRIPTION OF THE FIGURES**

Figure 1 A: Shows the infarct size to risk ratio (%) in normoxic hearts in the absence and presence of various cardioprotective and cardiotoxic substances. Results are presented as infarct/risk percentages (I/R%). * = p<0.01 vs. control.

Figure 2 A: Shows a % cell death dose response curve (assessed using an MTT assay) in response to increasing concentrations to doxorubicin in adult cardiomyocytes.

Figure 2B: Shows a % cell death dose response curve (assessed using an MTT assay) in response to increasing concentrations to doxorubicin in the absence and presence of Cyclosporine A in neonatal cardiomyocytes.

Figure 3A: Shows the effect of various cardiotoxic substances on cardiac myocyte depolarisation in adult cardiomyocytes * p<0.05 vs. control. n=6.
Figure 3B: Shows the schematic representation of the depolarisation and hypercontraction and subsequently leading to apoptosis in cardiac myocytes. Laser stimulation on the confocal microscope leads to depolarisation of cardiac myocytes. This leads to reduced viability of the myocytes and hypercontracture is also observed.

Figure 3C: Shows the effect of cardiotoxic substances on cardiac myocyte hypercontracture in adult cardiomyocytes *p<0.05 vs. control. n=6.

Figure 4A: Shows the effect of various cardiotoxic substances on cardiac myocyte depolarisation in neonatal cardiomyocytes *p<0.05 vs. control. n=6.

Figure 4B: Shows the effect of cardiotoxic substances on cardiac myocyte hypercontracture in neonatal cardiomyocytes *p<0.05 vs. control. n=6.

Figure 5: miRNA expression levels for miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR-146a, miR-181 and miR-542-3p relative to the U6 small nuclear RNA as assessed by RT-PCR from RNA extracted from rat myocardium from naive control hearts or hearts subjected to 120 minutes of cardiotoxic drug Doxorubicin. miRNA expression was normalized to that of the control.

Figure 6: miRNA expression levels for miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR-146a, miR-181 and miR-542-3p relative to the U6 small nuclear RNA as assessed by RT-PCR from RNA extracted from rat myocardium from control hearts or hearts subjected to 20 minutes of cardiotoxic drug Doxorubicin. miRNA expression was normalized to that of the control.

Figure 7: miRNA expression levels for miR-21, miR-133a and miR-133b relative to the U6 small nuclear RNA in myocardium which was subjected to Doxorubicin treatment compared to naive myocardium over an acute 120 minutes time period as assessed by RT-PCR from RNA extracted from rat myocardium from control hearts or hearts subjected to 120 minutes of cardiotoxic drug Doxorubicin in the presence of MPTP inhibitor Cyclosporin A. miRNA expression was normalized to that of the control.
Figure 8: miRNA expression levels for miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR-146a, miR-181 and miR-542-3p relative to the U6 small nuclear RNA as assessed by RT-PCR from RNA extracted from rat myocardium from control hearts or hearts subjected to 120 minutes of cardiotoxic drug Doxorubicin. miRNA expression was normalized to that of the control.

Figure 9: miRNA expression levels for miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR-146a, miR-181 and miR-542-3p relative to the U6 small nuclear RNA as assessed by RT-PCR from RNA extracted from rat myocardium from control hearts or hearts subjected to 120 minutes of cardioprotective drug Cyclosporin A. miRNA expression was normalized to that of the control.

EXAMPLES

The present invention is further defined in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

The disclosure of each reference set forth herein is incorporated herein by reference in its entirety.

Example 1: Animal heart tissues and cells

Rat Model of Myocardial Infarction (1)

Animals were sacrificed by cervical dislocation. Hearts were rapidly excised and placed into ice-cold KH buffer. Hearts were quickly mounted onto the Langendorff system and retrogradely perfused with modified Krebs Henseleit (KH) bicarbonate buffer containing (in mmol) NaCl 118.5, NaHCO$_3$ 25, KCL 4.8, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.2, CaCl$_2$ 1.7, and glucose 12. KH buffer was gassed using 95% O$_2$ and 5% CO$_2$ and maintained at 37°C. The left atrium was trimmed away and a latex isovolumic balloon was carefully introduced into the left ventricle and inflated...
up to 5-10mmHg. Functional recording was via a physiological pressure transducer connected to a bridge amp and a Powerlab (AD Instruments Ltd. Chalgrove, UK). Left ventricular pressure (LVDP), heart rate and coronary flow were measured at regular intervals. To induce ischaemia a surgical needle was inserted under the left main coronary artery and the ends of the thread were passed through a tube to form a snare. Tightening of the snare induced regional ischaemia and releasing the thread initiated reperfusion. At the end of reperfusion, the snare was tightened to re-occlude the coronary artery branch.

Measurements of Infarct Area.

A solution of 0.25% Evans blue in saline was then infused slowly via the aorta to delineate the non risk zone of the myocardium, which stained dark blue. The hearts were weighed and frozen at -20°C. Frozen hearts were then sliced into 2mm thick transverse sections and incubated in triphenyltetrazolium chloride solution (1% in phosphate buffer) at 37°C for 10-12min and fixed in 10% Formalin, for at least 4 hours. Viable issue stained red and infarct tissue appeared pale. The risk zone and infarct areas were traced onto acetate sheets. Using computerised planimetry (Summsketch II, Summagraphics) the percentage of infarct tissue within the volume of the myocardium at risk was calculated (l/R%).

Experimental Protocol

All hearts were allowed to stabilise for 15 min prior to being subjected perfusion in the absence and presence of drug treatment for 120 min. One min before perfusion they were randomly distributed into the following groups:

a) hearts perfused with normal buffer (controls);

b) hearts perfused with normal buffer in the presence of test substance (i.e. doxorubicin),

c) hearts perfused in the buffer throughout the reperfusion period,

d) hearts perfused with substance (i.e. doxorubicin) in addition to Cyclosporin A, which was added in the buffer throughout the reperfusion period.

The results are as shown in Figure 1.

Example 2: Application of known cardiotoxic drugs to cardiomyocytes

These substances were applied to the cardiomyocyte preparations under normoxic conditions in the absence and presence of cardiotoxic drug doxorubicin in the presence and absence of potential adjunct therapy agent Cyclosporin A.

5 **Cell Isolation from Neonatal Rat Heart and Primary Cell Culture**

Neonatal rat ventricular cardiomyocytes were isolated and cultured. Briefly, 1-3 days old rats were decapitated and their hearts were aseptically removed. Their ventricles were dissected, minced and trypsinized overnight at 4°C. The next day, cells were dissociated with collagenase and pre-plated twice for 60 min at 37°C. The non-adherent cardiomyocytes were removed and plated in 24-well plates in DMEM/F-12 medium (Invitrogen) containing 10% FBS and 0.1 mM bromodeoxyuridine (Sigma). 1x105 cells/well were seeded in 24-well plate for further experiments. This procedure yielded cultures with 90-95% myocytes, as assessed by microscopic observation of cell beating.

15 **Isolated Adult Rat Myocyte Model**

Myocytes were isolated from adult male Sprague Dawley rat hearts using a modification of a previously described technique (Maddock et al., 2001, Circulation. 2001;103:31 11-31 16). Briefly, the heart was removed and mounted on a constant flow (13 ml/min) Langendorff-perfusion apparatus and perfused in sequence with (a) Ca²⁺-free isolation buffer (in mmol/L: NaCl 130; KCl 5; MgSO₄ 0.8; HEPES 20 mmol/L; NaH₂PO₄ 0.9; Glucose 5.6; BDM 30; EGTA 0.003; BSA 1 mg/ml) solution for 5 minutes, (b) collagenase solution (isolation buffer plus collagenase 1mg/ml and CaCl₂ 0.025 mmol/L) for 10 minutes and (c) calcium solution (in mmol/L: isolation buffer plus CaCl₂ 0.05) for 5 minutes. The heart underwent 3-4 further episodes of digestion with collagenase. The myocytes were then seeded on laminin-coated cover-slips and kept in restoration buffer (in mmol/L: BSA 10mg/ml; Na pyruvate 0.5; taurine 5.0; l-carnitine 2.0; creatine 1.0; CaCl₂ 0.075) for experiments on the same day or kept in medium containing 1.8 mmol/L Ca²⁺ (Medium 199, Sigma, M7653) and penicillin/streptomycin in a humidified incubator at 37°C overnight for experiments the next day. Cell viability was determined before and after every treatment protocol using a standard MTT assay (Slater, T. et al. (1963) Biochem. Biophys. Acta 77:383; van de Loosdrecht, A.A., et al. J. Immunol. Methods 174: 311-320, 1994; Alley, M.C., et al. Cancer Res. 48: 589-601, 1988.)

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in living cells(Mosmann, Tim (1983). *Journal Of Immunological*...
Methods 65 (1-2): 55-63. A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600nm) by a spectrophotometer. The absorption maximum is dependent on the solvent employed.

MTT assay

Following Isolation, the concentration of the cells were determined with the nucleo counter (Chemometec). Drug preparation was done by serial dilution of the stock drug with restoration buffer to the desired concentrations. 10 wells were used as control by aliquoting 100 µl of restoration buffer. The other wells were used for the treatment groups. 50 µl of cells containing 10000 cells were incubated with 50 µl of the desired concentration of the drug in a 96 well plate for two hours. 6 wells were used for each concentration of drug (5 incubation with the cells and 1 well was used for the drug alone). After 2 hours incubation with the drug, the cells were treated with 20 µl of 5mg/ml MTT and incubated for another two hours and covered with foil. Following MTT incubation, 100 µl of lysis buffer (containing SDS and dimethyl formamide) was added to each well and incubated overnight. The absorbance was read at 492nm wavelength.

The mean absorbance of each treatment group was obtained and the absorbance for the drug alone was subtracted from this. This was calculated as a percentage of the mean absorbance of the control group and plotted using Origen pro 8.1.

Experimental protocol

It was investigated whether certain substances are cardiotoxic or cardioprotective in the adult cardiac myocytes in the absence and presence of intracellular signalling inhibitor. The cells were incubated with test substance for 2h in the presence and absence of inhibitor test substance

a) myocytes incubated with normal buffer (controls);
b) myocytes incubated with normal buffer in the presence of test substance (i.e. doxorubicin);
c) myocytes incubated in the buffer throughout the reperfusion period;
d) myocytes incubated with substance (i.e. doxorubicin) added in the buffer throughout the reperfusion period;
e) myocytes incubated with substance (i.e. doxorubicin) in addition to Cyclosporin A, added in the buffer throughout the reperfusion period.
Statistical Analysis

All values will be expressed as mean ± SEM. Infarct size and myocyte data will be analysed by one-way ANOVA and Fisher’s protected least significant difference test for multiple comparisons. Differences will be considered significant when P<0.05.

The results are as shown in Figure 2 (A and B).

Example 3: Model for detecting mPTP opening in the intact cell

Adult and neonatal cardiomyocytes were isolated following procedures detailed in Example 2. Doxorubicin is known to have cardiotoxic effects. This toxic effect was demonstrated by using a model for detecting mPTP opening in neonatal and adult cardiomyocytes.

In order to open the mPTP routinely and predictably, we have used controlled intramitochondrial oxidative stress and have used mitochondrial membrane depolarisation and the rigor contracture that follows the resultant ATP depletion as endpoints. The fluorescent dye TMRM was used: (a) to report mitochondrial membrane potential, with global mitochondrial depolarisation signalling mPTP opening, and (b) to act as a photosensitising agent to generate the oxidative stress on illumination required to induce mPTP opening followed by rigour contracture (Huser J., Blatter L.A.(1999) Biochem. J. 343(Pt. 2):31 1-317; Huser J., Rechenmacher C.E., Blatter L.A.(1998) J. 74(4):21 29-21 37; Duchen M.R., Leyssens A., Crompton M.(1998) J. Cell Biol. 142(4):975-988; Duchen M.R. (2000) Cell Calcium. 28(5-6):339-348; Jacobson J., Duchen M.R.(2002) J. Cell Sci. 115(Pt. 6):1 175-1 188; De Giorgi F., et al. (2002) FASEB J. 16(6):607-609). Laser-induced oxidative stress was continued until mPTP opening had been provoked, and then continued until rigour contracture took place. This model represents a widely-reported and reliable way to reproducibly induce the loss of mitochondrial potential, which has been unequivocally identified as a reflection of mPTP opening. TMRM, a lipophilic cation, accumulates selectively into mitochondria according to the mitochondrial membrane potential. On laser-induced photo-sensitisation, TMRM generates reactive oxygen species (ROS) from within the mitochondria, which provoke mPTP opening. As the oxidative stress generated at reperfusion also involves excess production of radical species from mitochondria, this represents a useful model for reoxygenation-induced cell injury. Cells were incubated with TMRM (3 µM) for 15 min at 37°C followed by washing. Opening of the mPTP results in mitochondrial membrane depolarisation, which is detected in this model as an increase in TMRM fluorescence intensity. The relatively high concentration of TMRM in the mitochondria causes auto-quenching of fluorescence, so that the fluorescence signal becomes...

Cells were assigned to: (1) control-incubation with 0.01% DMSO vehicle control for 20 min (n = 12); (2) incubation with cyclosporin A (1.0 µM, n = 12) for 20 min. (3) incubation with doxorubicin, in the presence or absence of cyclosporin A. We measured the time taken to the beginning of global mitochondrial depolarisation (represented by an increase in cytosolic TMRM fluorescence and indicates mPTP opening) and the time taken to the beginning of irreversible cell shortening (rigor contracture, signalling ATP depletion).

Confocal imaging and analysis

The cover-slip with adherent myocytes was placed in a chamber and mounted on the stage of a Zeiss 510 CLSM confocal microscope equipped with × 40 oil immersion, quartz objective lens (NA 1.3). The cells were illuminated using the 543-nm emission line of a HeNe laser. For all photo-sensitisation experiments, all conditions of the confocal imaging system (laser power, confocal pinhole, set to give an optical slice of 1 µm, pixel dwell time and detector sensitivity) were identical to ensure comparability between experiments. The fluorescence of TMRM was collected using a 585-nm long pass filter. Images were analysed using the Zeiss software (LSM 2.8)

The results are as shown in Figure 3 (A-C) and Figure 4 (A and B).

Example 4: miRNA isolation

The test substance was applied to the heart preparation under normoxic conditions as described in Example 1.

miRNA was isolated from myocardium that had been subjected to control normoxic conditions in the presence or absence of the test substances (i.e. Doxorubicin).

miRNA samples were extracted using commercial protocols for tissue sample handling and miRNA extraction.

Preparation of total RNA and small-RNA enriched samples

20-50mg of heart tissue, that was previously snap frozen in RNALater, was used. MiRNAs were isolated using mirVana™ miRNA Isolation kit (Ambion, Applied Biosystems, Austin, exas, USA). The samples were homogenized in Lysis/Binding buffer until the visible clumps were dispersed. 1/10 volume of miRNA Homogenate Additive (provided with the kit)
was added to the tissue lysate, vortexed and stored on ice for 10 minutes. The samples were then treated with a volume of Acid-Phenol :Chloroform that was equal to the tissue lysate before the addition of miRNA Homogenate Additive and vortexed for 1 minute. The Phenol-chloroform phase and the aqueous phases were separated by centrifugation (10 000 x g, room temp, 5 min).

1.25 volumes of 100% ethanol was added to the aqueous phase. The lysate/ethanol mixture were then filtered using a filter cartridge supplied with the kit and centrifuged at 10000g for 15 seconded. The wash through was discarded. 700 µL miRNA Wash Solution 1 was applied through the Filter Cartridge and centrifuged. After discarding the flow through, 500 µL Wash Solution 2/3 was applied through the Filter Cartridge and centrifuged. After repeating the previous procedure, the Filter Cartridge was placed in the same collection tube and centrifuged for 1 minute to remove the residual fluid. The RNA was recovered by transferring the Filter Cartridge into a fresh collection tube and applied with 100 µL of pre-heated (95 °C) Elution Solution. The eluate which contained the RNA was collected and stored -EO°C.

The sample concentration were measured by spectrophotometry (NanoDrop Technology, Delaware, USA) and RNA 6000 Nano (Agilent 2100 Bioanalyser; Agilent Technologies).

To assess RNA quality 1µL of RNA (~250ng) was analysed using Agilent Bioanalyser RNA 6000 assay. The RNA electrodes were first cleaned with RNA RNAZap (Ambion, Huntington, UK) and water.

Samples and RNA 6000 Ladder were heat denatured (70 °C for 2 min) and placed on ice until use. Filtered gel matrix was prepared by adding 550µL of RNA matrix into a spin filter and centrifuging at 1500 x g for 10 min. 65µL of this was aliquoted into RNase-free tubes and placed on ice. 1µL of RNA dye was added to a 65µL aliquot of filtered gel matrix and centrifuged at 13,000 x g for 10 min. 9µL of this gel dye mix was used to prepare the RNA 6000 nanochip in the chip priming station (in well C4). After priming the chip, another 9µL was added into each well of the chip marked with a G (A4 and B4). This was followed by adding 5µL of RNA 6000 Nano Marker into the remaining 13 wells of the chip. 1µL of each sample and 1µL of RNA 6000 Ladder were added to the wells on the chip. The chip was then vortexed for 1 min and used for the Eukaryotic total RNA Nano assay.

Example 5: Analysis of miRNA expression

miRNA expression was analysed from myocardium that had been subjected to control normoxic conditions in the presence or absence of the test substances (i.e. Doxorubicin).
Real-time RT-PCR (qPCR)

Mature miRNA expression levels were quantified using TaqMan® MicroRNA Assays (Applied Biosystems). Stem-loop primers were used for reverse transcription (RT) and this was followed by q-PCR. For RT, each reaction contained 100 mM dNTPs, 50U of MultiScibe™ Reverse Transcriptase, 3.76U of RNase Inhibitor, 3µl of RT-primer and 5ng of small RNA sample. Reactions were incubated for 30 min at 16°C, 30 min at 42°C and 5 min at 85°C. qPCR reaction mix was prepared by adding 10µl of TaqMan® Fast Advanced Master Mix (2X), 1µl of TaqMan® Gene Expression Assay (20 X), 2µl of cDNA prepared in RT and 7µl of nuclease-free water. The reaction was incubated for 10 min at 95°C followed by 40 cycles of 15 seconds at 95°C, 60 seconds at 60°C, with data collection at end of each cycle.

All RT reactions were performed with three different RNA inputs, and all PCR reactions were carried out in triplicate at least.

U6snRNA was used as an internal control. The measured Ct values were determined and the magnitude of the variance did not warrant DDCt normalization.

Fold variations in expression of miRNAs (i.e. miR-1) between RNA samples in comparison to the naive control were calculated.

The results are shown in Figures 5 and 6. The results show that miRNA-1, miRNA-21, miRNA-27a, miRNA133a, miRNA133b, miRNA146a, miRNA-181 and miRNA-542-3p are differentially expressed when cardiotoxic substances such as Doxorubicin are applied to rat myocardium

**Upregulated miRNAs after 20 minutes**

(1) miR-542-3p (SEQ ID No: 8)

This miRNA is upregulated after acute administration for 20 minutes of cardiotoxic agent doxorubicin (0.85-fold) in rat hearts.

**Upregulated miRNAs after 120 minutes**

(2) miR-1 (SEQ ID No: 1)

This miRNA is upregulated after acute administration for 120 minutes of cardiotoxic agent doxorubicin (3.38 fold) in rat hearts.

(3) miR-27a (SEQ ID No: 3)

This miRNA is upregulated after acute administration for 120 minutes of cardiotoxic agent doxorubicin (5.48-fold) in rat hearts.

(4) miR-146a (SEQ ID No: 6)
This miRNA is upregulated after acute administration for 120 minutes of cardiotoxic agent doxorubicin (0.13-fold) in rat hearts.

(5) miR-542-3p (SEQ ID No: 8)
This miRNA is upregulated after acute administration for 120 minutes of cardiotoxic agent doxorubicin (0.86-fold) in rat hearts.

**Downregulated miRNAs after 20 minutes**

(6) miR-21 (SEQ ID No: 2)
This miRNA is downregulated after acute administration for 20 minutes of cardiotoxic agent doxorubicin (0.89-fold) in rat hearts.

(7) miR-133a (SEQ ID No: 4)
This miRNA is downregulated after acute administration for 20 minutes of cardiotoxic agent doxorubicin (2.54-fold) in rat hearts.

(8) miR-133b (SEQ ID No: 5)
This miRNA is downregulated after acute administration for 20 minutes of cardiotoxic agent doxorubicin (1.88-fold) in rat hearts.

(9) miR-181 (SEQ ID No: 7)
This miRNA is downregulated after acute administration for 20 minutes of cardiotoxic agent doxorubicin (0.1-fold) in rat hearts.

**Downregulated miRNAs after 120 minutes**

(10) miR-21 (SEQ ID No: 2)
This miRNA is downregulated after acute administration for 120 minutes of cardiotoxic agent doxorubicin (0.18-fold) in rat hearts.

(11) miR-133a (SEQ ID No: 4)
This miRNA is downregulated after acute administration for 120 minutes of cardiotoxic agent doxorubicin (1.36-fold) in rat hearts.

(12) miR-133b (SEQ ID No: 5)
This miRNA is downregulated after acute administration for 120 minutes of cardiotoxic agent doxorubicin (0.68-fold) in rat hearts.

Example 6: Testing of adjunct test substances

Animal Langendorff hearts are prepared as described in Examples 1. The test substance was applied to the heart preparation under normoxic conditions as described in Example 1. miRNA expression produced by the test substance was compared to the expression produced by known cardiotoxic drugs as described in Example 4 and 5. Fold variations in expression of miRNAs (i.e. miR-1) between RNA samples in comparison to the naive control were calculated. The results are shown in Figure 7 and 8. This shows that expression of miRNA-1, miRNA-21, miRNA-27a, miRNA-133a, miRNA-133b, miRNA-146a, miRNA-181 and miRNA-542-3p are differentially expressed when cardiotoxic substances Doxorubicin is applied to rat myocardium in the presence of adjunct therapy MPTP inhibitor Cyclosporin A.

Example 7: Testing of cardioprotective test substances

Animal Langendorff hearts are prepared as described in Examples 1. The test substance was applied to the heart preparation under normoxic conditions as described in Example 1. miRNA expression produced by the test substance was assessed as described in Example 4 and 5. Fold variations in expression of miRNAs (i.e. miR-1) between RNA samples in comparison to the naive control were calculated. The results are shown in Figure 9. This shows that expression of miRNA-1, miRNA-21, miRNA-27a, miRNA-133a, miRNA-133b, miRNA-146a, miRNA-181 and miRNA-542-3p are differentially expressed when cardioprotective substances Cyclosporin A is applied to rat myocardium.
CLAIMS

1. A method of obtaining an indication of the presence of subclinical myocardial injury in a subject, comprising the steps:

   (i) detecting and/or measuring the levels or amounts of one or more miRNAs produced in a first biological sample obtained from the subject in order to produce a miRNA profile of that first biological sample, wherein the miRNAs are selected from the group consisting of miR-27a, miR-1, miR-21, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p,

   (ii) comparing the miRNA profile of that first biological sample with one or more miRNA profiles produced from second biological samples obtained from one or more subjects which are

       (a) known to have subclinical myocardial injury, or

       (b) known not to have subclinical myocardial injury,

   in order to obtain an indication of the presence of subclinical myocardial injury in the subject.

2. A method of obtaining an indication of the presence of subclinical myocardial injury in a subject, comprising the step:

   (i) detecting and/or measuring a change in the level of one or more miRNAs produced in a first biological sample obtained from the subject compared to the corresponding miRNA level in a biological sample obtained from a control subject who is known not to have subclinical myocardial injury, wherein the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p, wherein an increase in the level of one or more of the miRNAs in the first sample from the subject compared to the control, which miRNA level has been shown to be increased in subjects which are known to have subclinical myocardial injury, is indicative of the presence of subclinical myocardial injury in the subject,

   and

   wherein a decrease in the level of one or more of the miRNAs in the first sample from the subject, which miRNA level has been shown to be decreased in subjects which are known to have subclinical myocardial injury, is indicative of the presence of subclinical myocardial injury in the subject.
3. A method as claimed in claim 2, wherein a decrease in the level of one or more of the following miRNAs relative to a control is indicative of myocardial injury, preferably subclinical cardiotoxic induced myocardial injury: miRNA-133a, miRNA-133b, miRNA-21, miRNA-181.

5. A method as claimed in claim 2, an increase in the level of one or more of the following miRNAs relative to a control is indicative of myocardial injury, preferably subclinical cardiotoxic induced myocardial injury: miRNA-1, miRNA-27a, miRNA-146a, miRNA-542-3p.

5. A method of screening for test compounds which are capable of reducing a cardiotoxic effect of a medicament, comprising the steps:
   (i) determining the level of one or more miRNAs which are produced by mammalian cells in the absence of the medicament,
   (ii) determining the level of one or more miRNAs which are produced by mammalian cells in the presence of the medicament,
   (iii) determining the level of one or more miRNAs which are produced by mammalian cells in the presence of the medicament and the test substance,
wherein steps (i), (ii) and (iii) are carried out under the same conditions,
wherein the miRNAs are selected from the group consisting of miR-27a, miR-1, miR-21, miR-133a, miR-133b, miR-146a, miR-181 and miR-542-3p,
wherein if the level of the miRNA which is determined in step (ii) increases compared to the level determined in step (i),
then test substances which are found to reduce the level of miRNA which is produced in Step (iii) compared the level of miRNA produced in Step (ii) are test substances which are capable of reducing a cardiotoxic effect of the medicament,
and wherein if the level of the miRNA which is determined in step (ii) decreases compared to the level determined in step (i),
then test substances which are found to increase the level of miRNA which is produced in Step (iii) compared the level of miRNA produced in Step (ii) are test substances which are capable of reducing a cardiotoxic effect of the medicament.

6. An *in vitro* method of obtaining an indication of the cardiotoxic or cardioprotective effect of a test substance, comprising the steps:
   (i) contacting mammalian cells with the test substance,
(ii) detecting and/or measuring the level of one or more miRNAs produced by the mammalian cells, wherein the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR-181 and/or miR-542-3p, in order to produce a miRNA profile for that test substance, and

(iii) comparing the miRNA profile of the test substance with one or more miRNA profiles produced by substances under the same conditions which have been shown to be cardiotoxic or cardioprotective, in order to obtain an indication of the cardiotoxic or cardioprotective effect of the test substance.

7. An in vitro method of obtaining an indication of the cardiotoxic or cardioprotective effect of a test substance, comprising the steps:

(i) contacting mammalian cells with the test substance,

(ii) measuring the level of one or more miRNAs produced by the mammalian cells, wherein the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR-181 and/or miR-542-3p, wherein an increase or decrease in the level of the miRNA produced by the mammalian cells compared to the level of the same miRNA produced by control mammalian cells in the absence of the test substance is indicative of the test substance having a cardiotoxic or cardioprotective effect.

8. An in vitro method of obtaining an indication of the cardiotoxic or cardioprotective effect of a test substance, comprising the steps:

(i) contacting mammalian cells with the test substance, and

(ii) detecting and/or measuring a change in the production of one or more miRNAs by the mammalian cells compared to control mammalian cells which have not been contacted with the test substance under the same conditions, wherein the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR-146a, miR-181 and/or miR-542-3p, wherein an increase in the level of one or more of the miRNAs whose level has been shown to be increased upon contact of the mammalian cells with a cardiotoxic substance under the same conditions is indicative of a cardiotoxic effect of the test substance, and wherein a decrease in the level of one or more of the miRNAs whose level has been shown to be decreased upon contact of the mammalian cells with a cardiotoxic substance under the same conditions is indicative of a cardiotoxic effect of the test substance,
wherein an increase in the level of one or more of the miRNAs whose level has been shown to be increased upon contact of the mammalian cells with a cardioprotective substance under the same conditions is indicative of a cardioprotective effect of the test substance, and wherein a decrease in the level of one or more of the miRNAs whose level has been shown to be decreased upon contact of the mammalian cells with a cardioprotective substance under the same conditions is indicative of a cardioprotective effect of the test substance.

9. A method as claimed in any one of claims 5 to 8, wherein a decrease in the level of one or more of the following miRNAs relative to a control is indicative of a cardiotoxic effect of the test substance: miRNA-133a, miRNA-133b, miRNA-21, miRNA-181.

10. A method as claimed in any one of claims 5 to 8, wherein an increase in the level of one or more of the following miRNAs relative to a control is indicative of a cardiotoxic effect of the test substance: miRNA-4, miRNA-27a, miRNA-146a, miRNA-542-3p.

11. A method as claimed in any one of claims 5 to 10, wherein the mammalian cells are mammalian heart or muscle cells.

12. A method as claimed in claim 11, wherein mammalian cells are myocytes, myoblasts or cardiomyocytes.

13. A method as claimed in claim 11, wherein the mammalian cells are adult and neonatal myocytes, human-derived cardiomyocyte stem cells, cardiomyocyte cell lines, skeletal muscle, rat skeletal muscle myoblasts, Giradi, H2C9 cells, C2C12 mouse C3H muscle myoblasts or HL-1 cells.

14. A method as claimed in any one claims 5 to 13, wherein the mammalian cells are in the form of a heart or muscle tissue sample.

15. A method as claimed in any one of claims 5 to 14, wherein the mammalian cells are representative of a specific patient population.

16. A method as claimed in claim 15, wherein the patient population is young subjects or aged subjects.
17. A method as claimed in any one of claims 5 to 16, wherein the mammalian cells are cultured under conditions which are representative of a specific pathological condition.

18. A method as claimed in claim 17, wherein the pathological condition is ischemia and/or reperfusion, or myocardial infarct.

19. A method as claimed in any one of claims 5 to 18, wherein the mammalian cells are cultured in a medium comprising a second test substance.

20. A method as claimed in claim 19, wherein the second test substance is a bronchodilator.

21. A method of obtaining an indication of the therapeutic or adverse effect of a test substance which has been administered to a subject, the method comprising the steps:

(i) detecting and/or measuring the levels or amounts of one or more miRNAs produced in a first biological sample obtained from the subject in order to produce a miRNA profile of that first biological sample, wherein the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and/or miR-542-3p,

(ii) comparing the miRNA profile of that first biological sample with one or more miRNA profiles produced in second biological samples obtained from one or more subjects who are known to have been administered with therapeutic or adverse substances, in order to obtain an indication of the therapeutic or adverse effect of the test substance.

22. A method of obtaining an indication of the therapeutic or adverse effect of a test substance which has been administered to a subject, the method comprising the step:

(i) detecting the production or level of one or more miRNAs which are produced in a biological sample obtained from the subject, wherein the miRNAs are selected from the group consisting of miR-1, miR-21, miR-27a, miR-133a, miR-133b, miR146a, miR-181 and miR-542-3p,

wherein the production of one or more of miRNAs which have been shown to be produced in biological samples from subjects to whom a therapeutic substance has been administered is indicative of a therapeutic effect of the test substance, and
wherein the production of one or more miRNAs which have been shown to be produced in biological samples from subjects to whom a substance having a known adverse effect has been administered is indicative of an adverse effect of the test substance.

23. A method as claimed in claim 21 or 22, wherein (a) the adverse effect is a toxic or cardiotoxic effect or (b) the therapeutic effect is a cardioprotective effect.

24. A method of obtaining an indication of the susceptibility of a subject to a cardiac disease or disorder, the method comprising the steps:

(i) detecting and/or measuring the levels or amounts of one or more miRNAs produced in a first biological sample obtained from the subject in order to produce a miRNA profile of that first biological sample;

(ii) comparing the miRNA profile of that first biological sample with one or more miRNA profiles produced in second biological samples obtained from one or more subjects which are

(a) known to be susceptible to a cardiac disease or disorder, or

(b) known to be not susceptible to a cardiac disease or disorder,

in order to obtain an indication of the susceptibility of a subject to a cardiac disease or disorder.

25. A method of obtaining an indication of the susceptibility of a subject to a cardiac disease or disorder, the method comprising the steps:

(i) detecting the production of one or more miRNAs which are produced in a biological sample obtained from the subject,

wherein the production of one or more miRNAs which are known to be produced in biological samples from subjects which are known to be susceptible to a cardiac disease or disorder is indicative of the susceptibility of the subject to a cardiac disease or disorder.

26. A method of obtaining an indication of the susceptibility of a subject to a cardiac disease or disorder, the method comprising the steps:

(i) detecting and/or measuring a change in the production of one or more miRNAs produced in a first biological sample obtained from the subject compared to one or more miRNAs produced in a biological sample obtained from a control subject who is known to be not susceptible to a cardiac disease or disorder,
wherein an increase in the production of one or more miRNAs whose expression is known to be increased in subjects who are known to be susceptible to a cardiac disease or disorder is indicative of the susceptibility of the subject to a cardiac disease or disorder.

27. A method for obtaining an indication of the optimal treatment for a subject having a cardiac disease or disorder, the method comprising the steps:
   (i) detecting and/or measuring the levels or amounts of one or more miRNAs produced in a first biological sample obtained from the subject in order to produce a miRNA profile of that first biological sample;
   (ii) comparing the miRNA profile of that first biological sample with one or more miRNA profiles produced in one or more second biological samples obtained from one or more subjects prior to their successful treatment for a cardiac disease or disorder, in order to obtain an indication of the optimal treatment for the subject having a cardiac disease or disorder.

28. A method for obtaining an indication of the optimal treatment for a subject having a cardiac disease or disorder, the method comprising the steps:
   (i) detecting the production of one or more miRNAs which are produced in a biological sample obtained from the subject,
wherein the production of one or more miRNAs which are known to be produced in biological samples from subjects which were subsequently successfully treated for a cardiac disease or disorder is indicative of that treatment for the subject having a cardiac disease or disorder.

29. A method for obtaining an indication of the optimal treatment for a subject having a cardiac disease or disorder, the method comprising the steps:
   (i) detecting and/or measuring a change in the production of one or more miRNAs produced in a first biological sample obtained from the subject compared to one or more miRNAs produced in a biological sample obtained from a control subject, wherein an increase in the production of one or more miRNAs whose expression is known to be increased in biological samples from subjects who were subsequently successfully treated for a cardiac disease or disorder is indicative of that treatment for the subject having a cardiac disease or disorder, and
wherein a decrease in the production of one or more miRNAs whose expression is known to be decreased in biological samples from subjects who were subsequently successfully treated for a
cardiac disease or disorder is indicative of that treatment for the subject having a cardiac
disease or disorder.

30. A method for obtaining an indication of the prognosis of a subject having a cardiac
disease or disorder, the method comprising the steps:
   (i) detecting and/or measuring the levels or amounts of one or more miRNAs produced
   in a first biological sample obtained from the subject in order to produce a miRNA profile
   of that first biological sample;
   (ii) comparing the miRNA profile of that first biological sample with one or more miRNA
   profiles produced in one or more second biological samples obtained from one or more
   subjects previously having a cardiac disease or disorder,
   in order to obtain an indication of prognosis for the subject having a cardiac disease or disorder.

31. A method for obtaining an indication of the prognosis of a subject having a cardiac
disease or disorder, the method comprising the steps:
   (i) detecting the production of one or more miRNAs which are produced in a biological
   sample obtained from the subject,
   wherein the production of one or more miRNAs which are known to be produced in biological
   samples from subjects previously having a cardiac disease or disorder is indicative of a decline
   in the prognosis of the subject having a cardiac disease or disorder.

32. A method for obtaining an indication of the prognosis of a subject having a cardiac
disease or disorder, the method comprising the steps:
   (i) detecting and/or measuring a change in the production of one or more miRNAs
   produced in a biological sample obtained from the subject compared to one or more
   miRNAs produced in a biological sample obtained from a control subject,
   wherein an increase in the production of one or more miRNAs whose expression is known to be
   increased in biological samples from subjects having a more-advanced form of that cardiac
   disease or disorder is indicative of a detrimental progression or outcome of the disease or
   disorder in that subject.

33. A method for obtaining an indication of the exposure of a subject to a toxic substance,
the method comprising the steps:
(i) detecting and/or measuring the levels or amounts of one or more miRNAs produced in a first biological sample obtained from the subject in order to produce a miRNA profile of that first biological sample;

(ii) comparing the miRNA profile of that first biological sample with one or more miRNA profiles produced in second biological samples obtained from one or more subjects who are known to have been exposed to one or more toxic substances, in order to obtain an indication of the exposure of the subject to a toxic substance.

34. A method for obtaining an indication of the exposure of a subject to a toxic substance, the method comprising the steps:

(i) detecting the production of one or more miRNAs which are produced in a biological sample obtained from the subject,

wherein the production of one or more miRNAs which are known to be produced in biological samples from subjects who are known to have been exposed to one or more toxic substances is indicative of the exposure of the subject to a toxic substance.

35. A method for obtaining an indication of the exposure of a subject to a toxic substance, the method comprising the steps:

(i) detecting and/or measuring a change in the production of one or more miRNAs produced in a first biological sample obtained from the subject compared to:

(a) one or more miRNAs produced in a biological sample obtained from a control subject, or

(b) one or more miRNAs produced in a second biological sample obtained from the subject before exposure of the subject to the toxic substance,

wherein an increase in the production of one or more miRNAs whose expression is known to be increased upon exposure of a subject to a toxic substance is indicative of exposure of the subject to a toxic substance, and

wherein a decrease in the production of one or more miRNAs whose expression is known to be decreased upon exposure of a subject to a toxic substance is indicative of exposure of the subject to a toxic substance.

36. A method of screening for genes involved in one or more cardiac diseases or disorders, comprising the steps:
(i) identifying one or more miRNAs which are overexpressed in subjects suffering from one or more cardiac diseases or disorders compared to control subjects who are not suffering from one or more cardiac diseases or disorders, and

(ii) identifying the target genes to which the miRNAs from step (i) bind,

wherein the target genes to which the miRNAs from step (i) bind are ones which are involved in one or more cardiac diseases or disorders.

37. A method as claimed in any one of claims 24 to 36, wherein the miRNAs are selected from the group consisting of miR-27a, miR-1, miR-21, miR-133a, miR-133b, miR-146a, miR-181 and miR-542-3p,

...
Fig. 1

Effects of Doxorubicin on Ischaemia Reperfusion Injury

* p<0.05 vs IR Control
** p<0.01 vs IR Control
## p<0.01 vs Dox(100nM)IR
### p<0.01 vs Dox(1μM)IR

SUBSTITUTE SHEET (RULE 26)
Fig. 2A

<table>
<thead>
<tr>
<th>Model</th>
<th>DoseResp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equation</td>
<td>$y = A1 + (A2-A1)(1 + 10^{((\text{LOGx0-x)}*p)})$</td>
</tr>
<tr>
<td>Reduced Chi-Sqr</td>
<td>3.59595</td>
</tr>
<tr>
<td>Adj. R-Square</td>
<td>0.97567</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin A1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Doxorubicin A2</td>
<td>85.23417</td>
<td>1.10634</td>
</tr>
<tr>
<td>Doxorubicin LOGx0</td>
<td>-3.77921</td>
<td>0.06558</td>
</tr>
<tr>
<td>Doxorubicin P</td>
<td>-0.77771</td>
<td>0.10308</td>
</tr>
<tr>
<td>Doxorubicin Span</td>
<td>85.23417</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin EC20</td>
<td>9.88391E-4</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin EC50</td>
<td>1.66259E-4</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin EC80</td>
<td>2.79668E-5</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2B

- Dox
- Dox + CsA
- DoseResp Fit of Sheet1 Dox
- DoseResp Fit of Sheet1 Dox + CsA

% Cell Growth Compared to Control vs. Concentration (Log)
**Fig. 3A**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time to Depolarisation (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>250</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>150</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>350</td>
</tr>
<tr>
<td>Doxorubicin + Cyclosporin A</td>
<td>#</td>
</tr>
</tbody>
</table>

* p<0.05 vs. Control  
* # p<0.05 vs. Doxorubicin  
* ### p<0.001 vs. Doxorubicin

**Fig. 3B**

- Adult Cardiomyocyte
- Laser Stimulation
- Onset of Depolarisation
- Fully Depolarised
- Hypercontracture

**Fig. 3C**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time to Hypercontracture (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>600</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>500</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>700</td>
</tr>
<tr>
<td>Doxorubicin + Cyclosporin A</td>
<td>600</td>
</tr>
</tbody>
</table>

* ** p<0.01 vs. Control  
* *** p<0.001 vs. Control
Fig. 4A
Neonatal Rat Cardiomyocytes - Hypercontracture

** p<0.01 vs. Control
*** p<0.001

Fig. 4B
Neonatal Rat Cardiomyocyte - Depolarisation

* p<0.05 vs. Control
** p<0.01 vs. Control
Fig. 7

Average Fold Change Compared with Control

miRNA-133a miRNA-133b miRNA-21

- Dox
- CSA
- Dox + CSA