In vitro detection of cardiotoxins or neurotoxins affecting ion channels or pumps using beating cardiomyocytes as alternative for animal testing

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Abstract

The present study investigated if and to what extent murine stem cell-derived beating cardiomyocytes within embryoid bodies can be used as a broad screening in vitro assay for neurotoxicity testing, replacing for example in vivo tests for marine neurotoxins. Effect of nine model compounds, acting on either the Na^+, K^+, or Ca^{2+} channels or the Na^+/K^+-ATPase pump, on the beating was assessed. Diphenhydramine, veratridine, isradipine, verapamil and ouabain induced specific beating arrests that were reversible and none of the concentrations tested induced cytotoxicity. Three K^+ channel blockers, amiodarone, clofilium and sematilide, and the Na^+/K^+-ATPase pump inhibitor digoxin had no specific effect on the beating. In addition, two marine neurotoxins i.e. saxitoxin and tetrodotoxin elicited specific beating arrests in cardiomyocytes. Comparison of the results obtained with cardiomyocytes to those obtained with the neuroblastoma neuro-2a assay revealed that the cardiomyocytes were generally somewhat more sensitive for the model compounds affecting Na^+ and Ca^{2+} channels, but less sensitive for the compounds affecting K^+ channels. The stem cell-derived cardiomyocytes were not as sensitive as the neuroblastoma neuro-2a assay for saxitoxin and tetrodotoxin. It is concluded that the murine stem cell-derived beating cardiomyocytes provide a sensitive model for detection of specific neurotoxins and that the neuroblastoma neuro-2a assay may be a more promising cell-based assay for the screening of marine biotoxins.

Introduction

During the last two decades, the in vitro embryonic stem cell test (EST), in which murine D3 cells are induced to differentiate into beating cardiomyocytes formed in attached embryoid bodies (EBs), has been successfully implemented and validated by the European Centre for the Validation of Alternative Methods (ECVAM) for the assessment of embryo toxicity [1]. Where embryo toxicity is assessed by the effect of a compound on the differentiation process, the present study uses the beating cardiomyocytes within EBs as a model to study effects of neurotoxic compounds. The mechanism behind the contractions of the cardiomyocytes involves Na^+, Ca^{2+}, K^+ channels and the Na^+/K^+-ATPase pump. These channels and pump are also involved in the generation of the action potential in neuronal cells and are important for a wide range of physiological processes, including intracellular messaging, regulation of cell volume, regulation of gene expression, synaptic transmission, and cardiac excitation-contraction coupling [2]. The blockade or opening of these ion channels results in a disturbance of the ion homeostasis that in turn affects the physiology and action potentials of the cells. Figure 4.1 describes which channels are involved in the generation of action potentials both in cardiac and neuronal cells [3]. Although action potentials in the heart (both in pacemaker cells and in cardiomyocytes) and neurons are driven by similar ionic fluxes (Na^+, K^+ and Ca^{2+}), different ion channel subtypes exist, resulting in different specificity and sensitivity towards different neurotoxins [4]. However, despite the differences, murine embryonic stem cell-derived beating cardiomyocytes might be a promising model for the detection of neurotoxic compounds, including marine neurotoxins, because Na^+, Ca^{2+} and K^+ channels and the Na^+/K^+-ATPase pump are the target of a wide range of neurotoxic compounds [5-7]. Marine biotoxins are naturally occurring chemicals produced by microscopic algae. They accumulate in fish and shellfish and therefore represent a threat for consumers. The current standard for the detection of marine biotoxins in seafood is the in vivo mouse bioassay (MBA), in which mice are injected with sample extracts and death is the final readout. More recently, a chemical analytical LC/MS-MS method was EU approved/accepted but many countries still use the MBA, as the chemical analysis method is not able to detect all known toxins and misses unknown toxins. Extensive information on marine biotoxins can be found in recent reviews [8, 9]. In line with the 3R concept of Russell and Burch, alternative in vitro assays to replace in vivo testing are urgently needed as in vivo tests are considered as

highly unethical [10]. In the case of marine biotoxins, the mouse test will be forbidden from 2015 onwards, except for the control of production areas where seafood produced is intended for future consumption [11].

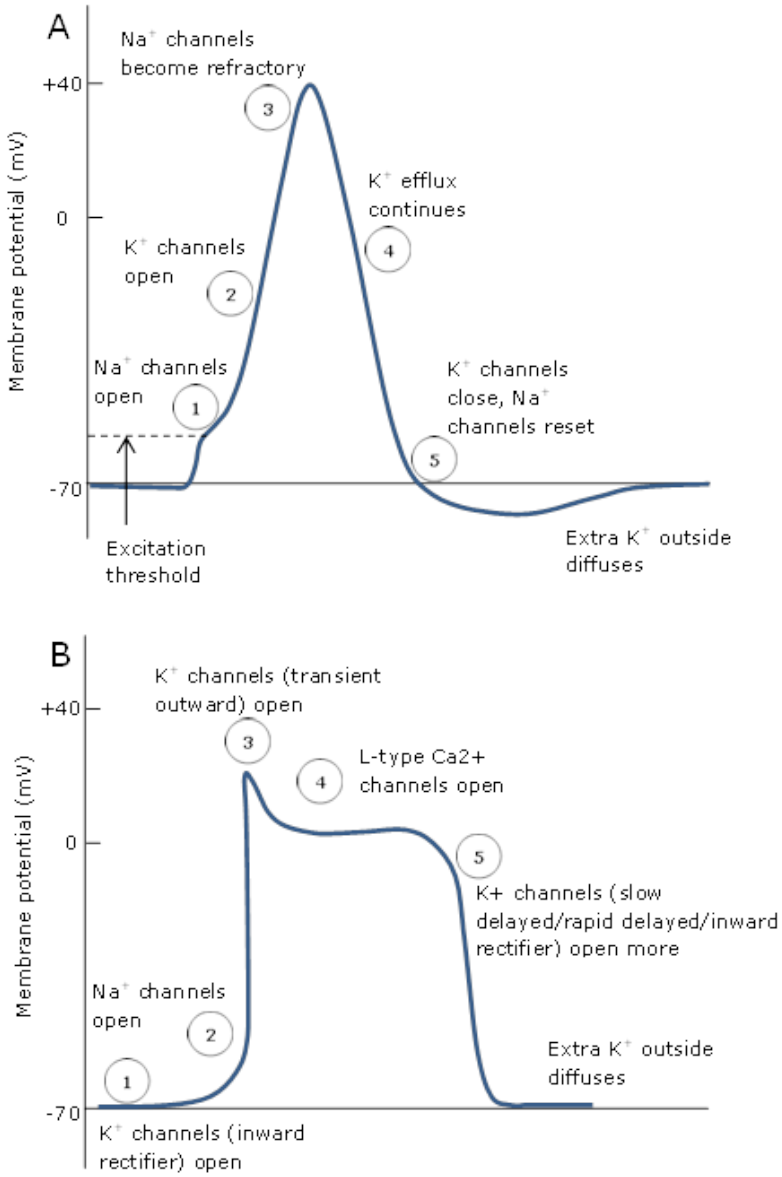

Figure 4.1: Overview of ion channels and fluxes involved in the generation of action potentials in A) neuronal cells and B) cardiomyocytes (based on [2, 3]). A) A stimulus increases the membrane potential above the excitation threshold until +40 mV thanks to Na+ influx. At +40 mV an efflux of K^+ ions brings the membrane potential back to -70 mV. B) K+ and Na+ influxes raise the membrane potential from -70 to +20 mV. Na+ channels close and L-type Ca2+ channels open, causing a plateau. K+ channels open decreasing the membrane potential back to its initial value of -70 mV.

Maltsev et al. (1994) showed that all basic cardiac-specific channels are present in murine embryonic stem cell-derived cardiomyocytes [12]. Since neurons and cardiomyocytes share many ion channels [13] and a wide range of neurotoxic compounds are known to act on ion channels, we hypothesized that beating cardiomyocytes might be suitable as an in vitro tool to detect the potential neurotoxic effects of compounds, including marine neurotoxins. Based on these considerations the aim of the present study was to assess whether and to what extent embryonic stem cell-derived cardiomyocytes could be used as an in vitro assay for the screening of neurotoxic compounds. To this end cardiomyocytes formed in EBs were exposed to different neurotoxins, using the contractility (i.e. the inotropy) of the cardiomyocytes within the EBs as a read-out. Nine model neurotoxins were selected that are known to affect either one of the three types of channels or the Na^+/K^+-ATPase pump (Tab. 4.1). In addition to these nine model neurotoxins, two commercially available pure marine neurotoxins were tested: saxitoxin (STX) and tetrodotoxin (TTX) (Na^+ channel blockers) in order to establish whether this assay may be of value as a replacement of the in vivo assays currently used for the screening of marine biotoxins in seafood. Moreover, the nine model compounds and STX and TTX were also tested in the neuroblastoma neuro-2a assay in order to compare the sensitivity of the embryonic stem cell-derived cardiomyocytes to the sensitivity of the neuro-2a cells, as the latter is currently regarded as a promising cell-based assay for the screening of marine biotoxins in seafood [8, 14].

Table 4.1. Inhibition of beatings in contractile embryonic stem cell-derived cardiomyocytes and effect on the viability in the neuro-2a assay.

Compound	Mode of action	References[a]	% cell viability at the noted drug concentration (differentiated	EC_{50} differentiated cardiomyocytes[c]	EC_{50} neuro-2a[c]
Diphenhydramine	Na^+ channel blocker	[15, 16]	150 µM: 104 ± 14	45 µM	> 100 µM
Veratridine	Na^+ channel opener	[17, 18] (EC_{50}: 85 µM)	100 µM: 114 ± 6	35 µM	90 µM
Isradipine	Ca^{2+} channel blocker	[19]	100 µM: 113 ± 23	15 µM	> 100 µM
Verapamil	Ca^{2+} channel blocker	[20, 21] (EC_{50}: 19 µM)	1 µM: 106 ± 10	100 nM	190 nM
Sematilide	K^+ channel blocker	[22]	500 µM: 95 ± 10	> 400 µM	> 300 µM
Clofilium	K^+ channel blocker	[23]	500 µM: 51 ± 0.25	207 µM due to cytotoxicity	150 µM
Amiodarone	K^+ channel blocker	[24]	60 µM: 54 ± 1	> 60 µM	80 µM
Ouabain	Na^+/K^+ ATPase blocker	[25] (EC_{50}: 370 µM)	600 µM: 86 ± 4	257 µM	220 µM
Digoxin	Na^+/K^+ ATPase inhibitor	[26]	160 µM: 64 ± 4	> 150 µM	> 100 µM
Saxitoxin	Na^+ channel blocker	[27]	No cytotoxicity up to 1 µM	4 µM	With o/v: 11 nM
Tetrodotoxin	Na^+ channel blocker	[28]	No cytotoxicity up to 10 µM	10 µM (with veratridine)	With o/v: 10 nM

[a]The references stand for the modes of action of the selected model compounds and the EC_{50} values are mentioned when available. [b]The percentage of cell viability was determined at concentrations above those which elicited effects on beatings of cardiomyocytes following a 24 hour exposure to the compounds. [c]The EC_{50} values were calculated using a non-linear regression model. o/v: ouabain/veratridine.

Materials and Methods

Chemicals

Amiodarone, clofilium, digoxin, diphenhydramine, isradipine, ouabain, sematilide, verapamil, and veratridine were purchased from Sigma-Aldrich (St. Louis, USA) and dimethylsulfoxide (DMSO) was obtained from Acros Organic (New Jersey, USA). STX was purchased from the National Research Council (Montreal, Canada) and TTX from Latoxan (Valence, France). Compound stock solutions were prepared in DMSO.

Cell lines and cell culture

The murine-derived embryonic stem cell line D3 was kindly donated by Johnson & Johnson (Beerse, Belgium). The cells were cultured in flasks of 25 cm^2 (Corning Inc., Cambridge, USA) in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Breda, The Netherlands) supplemented with 20% (v/v) heat inactivated fetal calf serum (FCS) (BioWhittaker, Maryland, USA), 1% (v/v) non-essential amino acids (Invitrogen), 50 U/mL penicillin/50 µg/mL streptomycin (P/S) (Invitrogen), 2 mM L-glutamine (Invitrogen) and 0.1 mM ß-mercaptoethanol (Sigma-Aldrich). To prevent cell differentiation, 1000 U/mL murine leukemia inhibitory factor (Sigma-Aldrich) was added to the medium when cells were seeded. The cells were cultured in a humidified atmosphere (37°C, 5% CO_2) and subcultured three times a week. The cells were detached when reaching 80% confluence using non-enzymatic dissociation buffer (Sigma-Aldrich).

Neuro-2a cells (LGC standards, Middlesex, UK) were cultured in flasks of 75 cm^2 using 10% FBS/Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 1% 5000 U/mL penicillin/5 mg/mL streptomycin (P/S) (Invitrogen), 2 mM L-glutamine (Invitrogen) and 1 mM sodium pyruvate (Sigma-Aldrich). Cells were cultured at 37°C, 5% CO_2 and subcultured 3 times per week. The cells were detached when reaching 80% confluence using trypsin (Sigma-Aldrich).

Embryonic stem cell test

The embryonic stem cell test was performed as described previously [29] with some minor modifications. For the differentiation process, hanging drops of 20 µl cell suspension ($3.75.10^4$ cells/mL) were prepared at day 0 on the cover of 96-well plates. Phosphate buffered saline (250 µL) was put in each well in order to maintain humidity and prevent evaporation of the hanging drops. Cells in hanging drops were allowed to differentiate for 3 days in the humidified atmosphere (37°C, 5% CO_2), and at day 3, the cell aggregates formed (called EBs) were transferred to bacterial petri dishes (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) and incubated for 3 days. At day 5, the EBs were plated in 96-well plates (Corning, 1 EB/well) and incubated for 5 days. The cardiomyocytes started beating on day 10.

At day 13, when most EBs have beating areas, beating EBs were incubated with the neurotoxic compounds for 1 h (37°C, 5% CO_2). Ten beating EBs per concentration were exposed to the different compounds selected (final DMSO solvent concentration of 0.25%) and the number of remaining beating EBs after one hour incubation was scored by visual inspection (beating arrest). Subsequently, cells were washed and the medium was replaced by medium without the test compound, and the EBs were incubated for an additional hour after which the contractility of the EBs was assessed again to evaluate recovery. At least three independent experiments with six replicates were performed per concentration for each compound. In the case of STX, five instead of ten EBs were exposed and the number of beating EBs were scored after 20 hours of incubation instead of one hour. The control for STX was a solution of HCl (0.003 M) as commercial STX is dissolved in HCl. Because TTX alone did not affect the beatings and TTX, being a sodium channel blocker, has an opposite mode of action as veratridine which acts as a sodium channel activator, two experimental designs were tested to evaluate whether TTX could prevent beating arrest following veratridine exposure: ten EBs were exposed to 10 µM of TTX for 5 minutes and then exposed to 100 µM of veratridine for one hour and in another experiment, ten EBs were exposed to 100 µM of veratridine for 1 h followed by an exposure to 10 µM of TTX for an additional hour.

Cell viability

Cell viability was assessed with the WST-1 assay by measuring mitochondrial activity (Roche, Woerden, The Netherlands) for embryonic stem cell-derived cardiomyocytes. The viability of the cardiomyocytes within EBs was assessed 24 hours after exposure to the different compounds and vehicle. To this end, 20 µL of WST-1 solution was added to each well containing 200 µL of medium. After incubation for 3 h (37°C, 5% CO_2), the absorbance was determined spectrophotometrically at 450 nm. The mitochondrial activity was expressed as percentage of the average of the vehicle control (DMSO solvent).

Neuro-2a assay

Cells were grown for 24 hours and subsequently exposed for 24 hours to the marine neurotoxins with and without a combination of ouabain/veratridine. Concentrations of ouabain/veratridine inducing about 80% cytotoxicity were selected, in order to evaluate the toxin's ability to oppose or prevent the cytotoxicity induced by this Na^+ channel opener (veratridine) and Na^+/K^+-ATPase pump blocker (ouabain). Cell viability of neuro-2a cells was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich) measuring the mitochondrial activity. Absorbance was read on a spectrophotometer at 570 nm and the mitochondrial activity was expressed as percentage of the average of the vehicle control (DMSO).

Data analysis

In this study, the EC_{50} of a compound is the concentration at which 50% of the maximum inhibiting effect on the contractility of the cardiomyocytes was induced. The EC_{50} values and their 95% confidence intervals (CI) were calculated using GraphPad Prism (San Diego, CA). When no inhibition of the contractility occurred, no EC_{50} value could be calculated (Tab. 4.1).

For the establishment of dose-response curves and the determination of EC_{50} values for the neuro-2a assay, cell viability for each concentration of each model compound or marine neurotoxin after a 24 hour exposure was measured at least in duplicate. In this assay, the EC_{50} is the concentration at which 50% of the cytotoxicity occurs following exposure to the model

compounds and the concentration at which 50% of the cytotoxic effect caused by ouabain/veratridine is opposed following exposure to the marine neurotoxins.

Results

With the embryonic stem cell test (EST), the proportion of beating EBs is the read-out and cell viability was assessed by the WST-1 assay as the MTT yielded unreliable results (due to loss of cells during the removal of the medium), while in the neuro-2a assay, the cell viability is the read-out and was assessed by the MTT assay as the neuro-2a cells were well attached to the bottom of each well. Moreover, in the neuro-2a MTT assay, STX and TTX and the nine neurotoxic model compounds were also tested in combination with a dose of ouabain/veratridine that caused a 80% decrease in cell viability in order to determine whether the toxin was able to oppose the effect, i.e. to counteract the cytotoxicity induced by ouabain/veratridine [14]. However, none of the nine neurotoxic model compounds was able to oppose the cytotoxicity induced by ouabain/veratridine, and these results are not further discussed or shown.

Na^+ channels

The Na^+ channel blocker diphenhydramine inhibited beating of cardiomyocytes at and above a concentration of 25 µM (EC_{50} = 45 µM, Tab. 4.1). A concentration of 100 µM elicited beating arrests of all EBs (Fig. 4.2A). This beating arrest was completely reversible, as upon refreshing the medium, all EBs started to beat again. Veratridine induced a concentration-dependent decrease of the proportion of beating EBs at concentrations at and higher than 25 µM (EC_{50} = 35 µM, Tab. 4.1). When the cells were exposed to 60 and 100 µM, all EBs stopped beating. After a recovery period of 1 h, every EB that stopped beating after exposure to 60 µM of veratridine started beating again, while for 100 µM 80% of the EBs recovered (Fig. 4.2B). The cell viability was not affected by both compounds according to the results obtained with the WST-1 assay (Tab. 4.1).

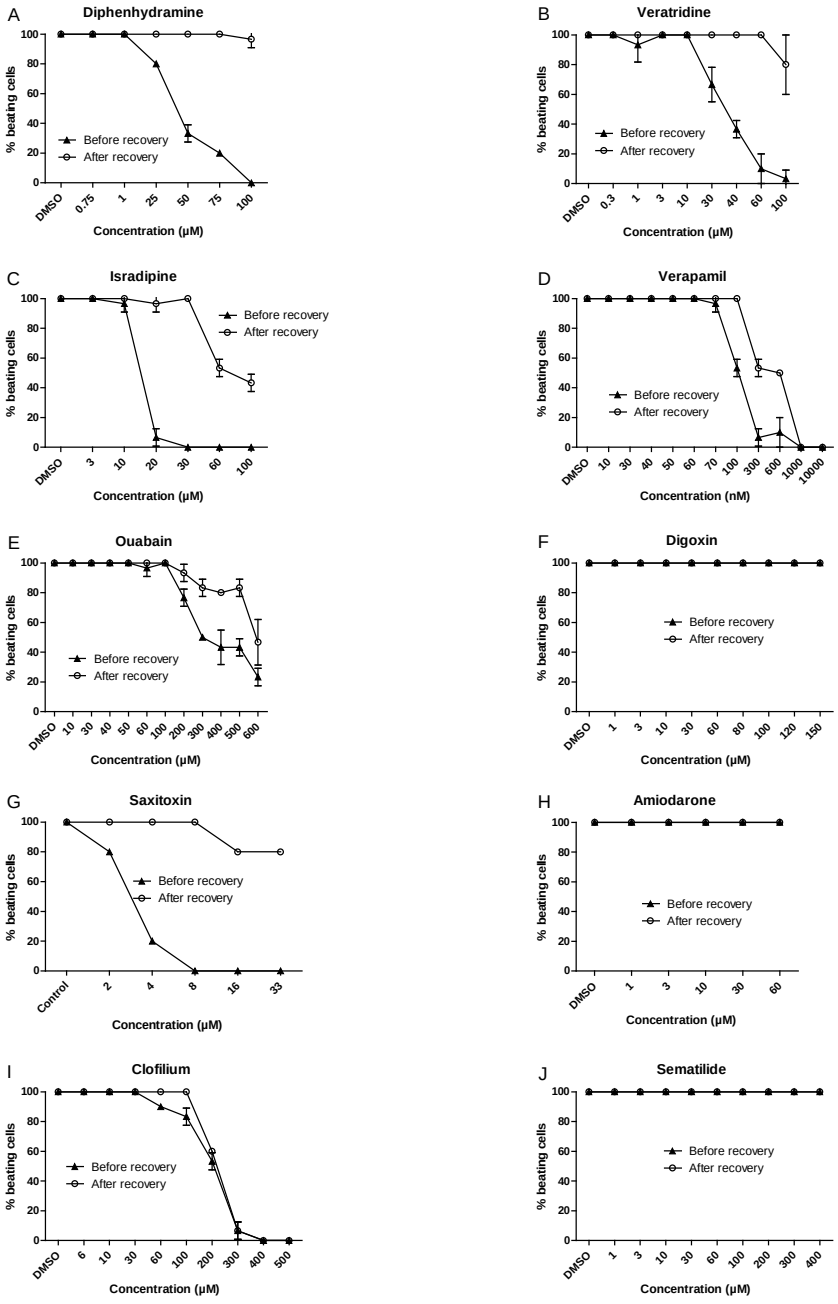

Figure 4.2: Effect of A) the Na^+ channel blocker diphenhydramine, B) the Na^+ channel opener veratridine, C-D) the Ca^{2+} channel blockers isradipine and verapamil, E-F) the Na^+/K^+-ATPase blockers ouabain and digoxin, G) the Na^+ channel blocker STX, H-I-J) the K^+ channel blockers amiodarone, clofilium and sematilide on the beating of embryonic stem cell-derived cardiomyocytes. Upon exposure the EBs were incubated with fresh medium for an additional hour to assess the recovery. The data are presented as the mean of 3 experiments ± SD. 0.3% DMSO or HCl, used as solvent controls, had no effect on cell viability. At least three independent experiments with ten replicates were performed per concentration for each compound.

Figure 4.3A and 4.3B show that in the neuro-2a assay, diphenhydramine slightly decreased cell viability while veratridine induced a concentration-dependent decrease in cell viability (EC_{50} = 90 µM, Tab. 4.1).

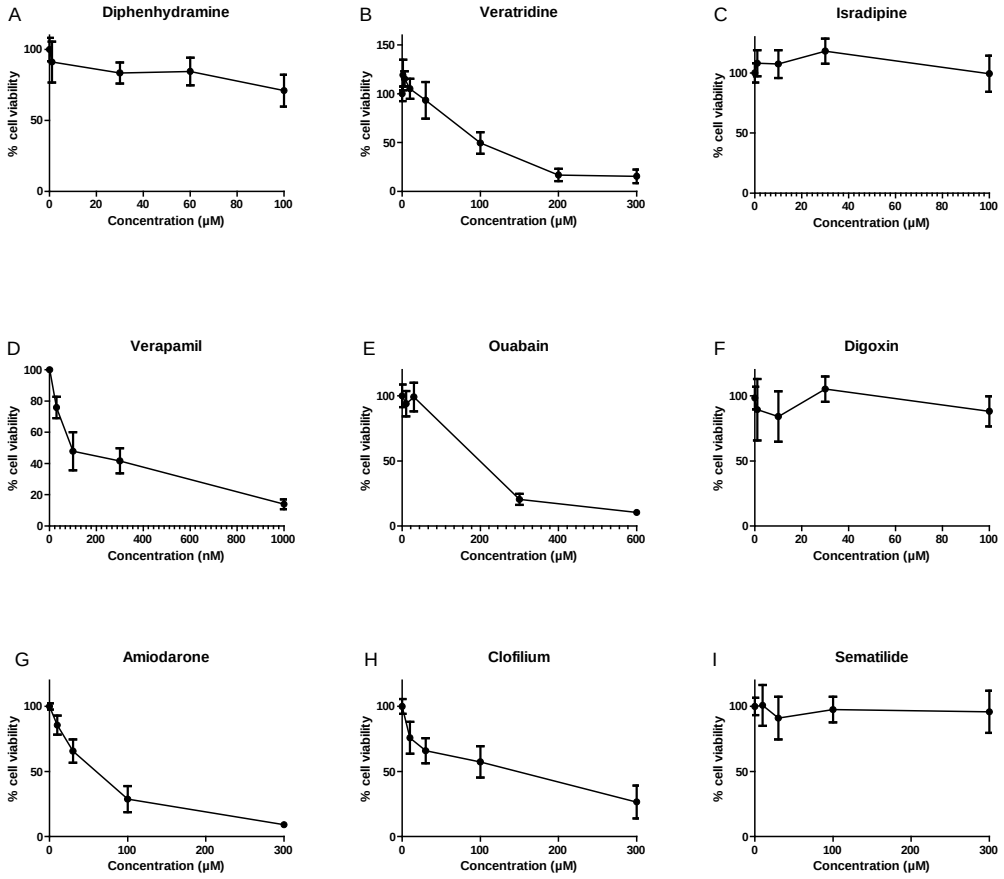

Figure 4.3: Effect of A) the Na^+ channel blocker diphenhydramine, B) the Na^+ channel opener veratridine, C-D) the Ca^{2+} channel blockers isradipine and verapamil, E-F) the Na^+/K^+-ATPase blockers ouabain and digoxin, G-H-I) the K^+ channel blockers amiodarone, clofilium and sematilide on the cell viability of neuro-2a cells. The cells were exposed for 24 hours and cell viability was assessed using the MTT assay. At least three independent experiments with six replicates were performed per concentration for each compound.

The embryonic stem cell-derived cardiomyocytes are thus more sensitive to diphenhydramine and veratridine than the neuro-2a cells.

Ca^{2+} channels

Figure 4.2C shows that the Ca^{2+} channel blocker isradipine at concentrations of 20 µM and higher caused beating arrests in every exposed EB (EC_{50} = 15 µM, Tab. 4.1). After exposure to up to 30 µM isradipine, all EBs recovered after refreshment of the medium. After exposure to 60 or 100 µM israpidine, 50% of the EBs recovered after medium refreshment. The other Ca^{2+} channel blocker, verapamil, induced beating arrests at concentrations from 100 nM onwards. Where 100 nM of verapamil induced beating arrest in half of the EBs, all EBs stopped beating at 300 and 1000 nM (EC_{50} = 100 nM, Tab. 4.1). All EBs recovered at 100 nM, while 50% recovered at 300 nM and 600 nM. At concentrations of 1000 nM and higher, the cells did not recover anymore (Fig. 4.2D). The WST-1 tests showed that cell viability was not affected by the tested concentrations of isradipine or verapamil (Tab. 4.1).

Figure 4.3C and 4.3D show that in the neuro-2a assay, isradipine up to 100 µM had no effect on the cell viability, while verapamil at concentrations of 30 nM and higher induced a decrease in cell viability.

The determined EC_{50} values show that the embryonic stem cell-derived cardiomyocytes are more sensitive for isradipine than neuro-2a cells, and also slightly more sensitive for verapamil (Tab. 4.1).

K^+ channels

None of the K^+ channel blockers induced beating arrests of the EBs at non-cytotoxic concentrations: amiodarone up to 60 µM, clofilium up to 100 µM and sematilide up to 400 µM had no effect on the beating (Fig. 4.2H-I-J). The cell viability was affected by amiodarone but not by sematilide according to the results obtained with the WST-1 assay (Tab. 4.1). However, higher concentrations of clofilium from 100 µM onwards induced cytotoxicity (WST-1 assay, Tab. 4.1), and the resulting beating arrests were thus not due to the K^+ channel blockade. In accordance with this observation, none of the EBs exposed to high levels of clofilium recovered (Fig. 4.2I).

In the neuro-2a assay, amiodarone and clofilium at and above a concentration of 10 µM induced a decrease in cell viability, while sematilide had no effect up to 300 µM (Fig. 4.3G-H-I).

The neuro-2a cells are thus more sensitive to amiodarone and clofilium than the embryonic stem cell-derived cardiomyocytes, while both cell assays were insensitive for sematilide (Tab. 4.1).

Na^+/K^+-ATPase pump

Figure 4.2E shows the effects of the Na^+/K^+-ATPase pump blocker ouabain on the beating of the cardiomyocytes. Concentrations up to 100 µM of ouabain did not induce a beating arrest in the EBs. At concentrations of 200 µM and higher, ouabain induced a concentration-dependent decrease in the percentage of beating cells (EC_{50} = 257 µM, Tab. 4.1). While most EBs recovered from the 300 µM exposure, almost none of the EBs that stopped beating at 600 µM recovered after the medium refreshment. It should be mentioned that the beating rates of some EBs that did not stop beating after exposure to ouabain (up to 100 µM) were largely increased (increased beating frequency not quantified) and this increased beating frequency may also reflect neurotoxicity. The Na^+/K^+-ATPase pump inhibitor digoxin did not affect the beating of the cells up to 150 µM (Fig. 4.2F). According to the outcomes of the WST-1 assay, the viability of the cardiomyocytes was not affected by ouabain but slightly affected by digoxin at 160 µM (Tab. 4.1).

Ouabain at concentrations of 300 µM and higher induced a decrease in cell viability of neuro-2a cells and digoxin up to 100 µM did not affect cell viability of neuro-2a cells (Fig. 4.3E and 4.3F). The two assays thus display a similar sensitivity towards these Na^+/K^+-ATPase pump inhibitors.

Marine biotoxins: Proof of principle

Figure 4.2G shows the effects of the commercially available Na^+ channel blocker STX on the beating of cardiomyocytes after 20 hours of exposure. As no beating arrest was observed after a 1 hour exposure to STX, the EBs were checked every hour for 6 hours and after 20 hours. STX only induced a concentration dependent decrease in the beating of the cardiomyocytes after a period of 20 hours (EC_{50} = 3.9 µM, Tab. 4.1). Every EB exposed to concentrations of STX up to 8 µM recovered, while 80% of them recovered at 16 and 33 µM. TTX up to 60 µM did not alter

the beating of cardiomyocytes after an exposure of 24 hours. Neither STX nor TTX had an effect on the viability of embryonic stem cell-derived cardiomyocytes (WST-1 assay, Tab. 4.1).

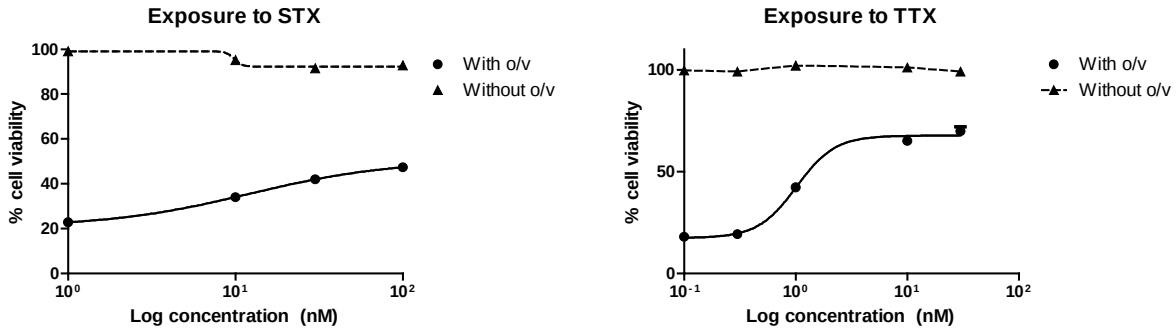

Figure 4.4: Effect of the Na^+ channel blockers saxitoxin and tetrodotoxin on the viability of neuro-2a cells, with (plain line) or without (dashed line) ouabain/veratridine. The cells were exposed to ouabain/veratridine and STX or TTX at the same time. The cell viability of the cells exposed to ouabain/veratridine alone was about 20%. No cytotoxicity was induced by STX and TTX without ouabain/veratridine (dashed line). At least three independent experiments with six replicates were performed per concentration for each compound.

Because TTX and veratridine have opposite modes of action, TTX being a Na^+ channel blocker and veratridine a Na^+ channel activator, it was hypothesized that TTX could prevent the beating arrest induced by veratridine. Indeed, exposing the cardiomyocytes to 100 µM of veratridine for 1 hour, resulting in a beating arrest of all EBs, and then adding TTX to a final concentration of 10 µM in each well during an additional hour, resulted in a 100% recovery of the beating. In addition, when the cells were pre-treated with 10 µM of TTX during 5 minutes and then exposed to 100 µM of veratridine for one hour, none of the EBs stopped beating.

Without ouabain/veratridine STX and TTX did not induce cytotoxicity in the neuro-2a cells. However, when co-incubated with ouabain/veratridine, STX and TTX increased cell viability in the neuro-2a cells at and above 10 nM and 3 nM respectively (Fig. 4.4). These data show that the neuro-2a cells are more sensitive for these marine biotoxins than cardiomyocytes (Tab. 4.1).

Discussion

Diphenhydramine has been reported to block Na^+ channels in neurons at a concentration of 100 µM [16]. Without a Na^+ influx, cardiac cells cannot generate action potentials and cardiomyocytes are therefore not able to beat. However, diphenhydramine did not decrease cell viability of neuro-2a cells, indicating that either neuro-2a cells do not express the Na^+ channel subtypes targeted by diphenhydramine or blockade of these channel subtypes does not affect cell viability.

The Na^+ channel opener veratridine causes an abnormal entry of Na^+ ions followed by a secondary increase of the Ca^{2+} concentration, leading to beating arrests because the Na^+ channel is unable to close [30]. The sensitivity of the murine embryonic stem cell-derived beating cardiomyocytes to veratridine in the present study, i.e. EC_{50} = 35 µM, is comparable to what has been found by Yanagita et al. in 2003, reporting an increase of Na^+ influx by veratridine with an EC_{50} of 85 µM in adrenal chromaffin cells [18]. The neuro-2a assays showed a similar sensitivity to veratridine, as cell viability was affected with an EC_{50} of 90 µM.

Verapamil blocks the L- and T-type Ca^{2+} channels, while isradipine blocks only the L-type Ca^{2+} channels [31, 32]. In 1994, Keith et al. obtained an EC_{50} of 19 µM for the synaptosomal inhibition of the Ca^{2+} influx by verapamil in rat cortical neurons [21]. The embryonic stem cell-derived beating cardiomyocytes represent a more sensitive assay for the detection of verapamil, as specific beating arrests were observed for verapamil with an EC_{50} of 100 nM, which is also lower than the one obtained with the neuro-2a assay (EC_{50} = 190 nM). This indicates that embryonic stem cell-derived cardiomyocytes cells are relatively sensitive to verapamil. The EC_{50} of isradipine (15 µM) for inducing beating arrests was much higher than that for verapamil, most likely because this compound only blocks the L-type Ca^{2+} channels. Isradipine had no effect on the cell viability of neuro-2a cells. Together these data suggest that the neuro-2a cells only express the T-type Ca^{2+} channels, making them sensitive to verapamil in the µM range, but do not express the L-type Ca^{2+} channels, making them insensitive to isradipine. Murine cardiomyocytes express both the L- and T-type Ca^{2+} channels and are therefore sensitive to both verapamil and isradipine.

The K^+ channel blockers amiodarone, clofilium and sematilide had no specific inhibiting effect on the beating cardiomyocytes. The K^+ channel isoforms on the surface of the murine

embryonic stem cell-derived cardiomyocytes might be resistant to amiodarone, clofilium and sematilide or, in the case of clofilium, the concentrations that elicited beating arrests in the murine cardiomyocytes are too close to the concentrations that elicited general cytotoxicity. The neuro-2a assay was also insensitive to sematilide, but amiodarone and clofilium were found to affect cell viability.

The Na^+/K^+-ATPase pump blocker ouabain elicited specific beating arrests and decreased cell viability of neuro-2a with a similar sensitivity. The EC_{50} of 257 µM obtained in the present study for ouabain is in line with the findings of Kagiava et al. [25] who showed that ouabain elicited neurotoxicity with an EC_{50} of 370 ± 18 µM in the mouse myelinated sciatic nerve fibres. Digoxin had no effect either on the beating (inotropy) of the cardiomyocytes in the EBs or on the cell viability of neuro-2a cells. This was expected as digoxin only decreases the function of the Na^+/K^+-ATPase pump and does not block it, However, digoxin appeared to affect beating frequency (chronotropy) in the cardiomyocytes which was not included as a read out in our test.

STX and TTX block Na^+ channels and therefore the cells cannot generate action potentials, reflecting a mode of action similar to that of diphenhydramine [33]. The incubation time with STX required to elicit beating arrests was 20 hours, while for all model neurotoxins tested one hour was sufficient. This difference may be due to the fact that STX may only partially block the Na^+ type channels expressed by the cardiomyocytes, resulting in residual amounts of Na^+ ions still entering the cells causing shortage of Na^+ and effects on the beating process only upon prolonged exposure. TTX did not have any effect on the beating up to 60 µM. However, none of the cardiomyocytes stopped beating with the combination of 10 µM TTX with 100 µM of the Na^+ channel opener veratridine, a concentration that stopped the beating of all EBs, indicating that co-exposure of the EBs to TTX with veratridine is suitable for detecting TTX. This finding is in line with the observation that TTX has been shown to block veratridine-induced effects [34]. STX or TTX had no effect on cell viability of neuro-2a cells without addition of ouabain/veratridine. However, neuro-2a cells swell and eventually lyse upon exposure to a combination of ouabain/veratridine that enhances sodium influx [35]. The Na^+ channel blocker STX has been shown to protect neuro-2a cells from the action of ouabain/veratridine [14]. Both STX and TTX prevented to a certain extent cytotoxicity induced by ouabain/veratridine in neuro-2a cells. This indicates that the main voltage gated sodium channels present in neuro-2a cells are

TTX-sensitive while in the cardiomyocytes these channels are less sensitive to TTX and most likely belong to the Nav1.5 channel subtypes [36].

Certain compounds affect channels that are not expressed on the surface of the murine cardiomyocytes such as the N- and R-type Ca^{2+} channels and will thus not be detected. Since K^+ channel blockers tested negative their integrity was confirmed by showing their activity towards other endpoints. Amiodarone inhibited neuronal activity in rat cortical neurons [37]. Measuring effects on the beating rate or on the generation of action potentials as additional parameters, using for example multielectrode arrays [38], will most likely result in a more sensitive assay and might even result in an assay able to detect a wider range of neurotoxins. Ultimately, a model capable of detecting an extensive range of marine neurotoxins shall present a large variety of ion channels/pumps as well as neuronal receptors, the principal targets of such toxins.

In line with the findings from Maltsev et al. (1994) the data provided in the present study show that the murine cardiomyocytes have functional Na^+, Ca^{2+} channels and Na^+/K^+-ATPase pump and that beating murine cardiomyocytes can be used as a model to detect specific neurotoxic effects of compounds on Na^+, Ca^{2+} channels and the Na^+/K^+-ATPase pump, but not on K^+ channels [12]. The inhibition of beatings in the murine cardiomyocytes was shown to be reversible and to occur at concentrations below those affecting cytotoxicity, which implies that these effects are not due to overall cytotoxicity providing a more specific read out for neurotoxicity than the cytotoxicity endpoint as determined in the neuro-2a assay. This is an advantage of the cardiomyocytes assay over the neuro-2a cells, providing a way to avoid detection of false positives for neurotoxicity. Moreover, for the nine neurotoxic model compounds tested, the cardiomyocytes were generally somewhat more sensitive for those compounds that affect Na^+ and Ca^{2+} channels, but less sensitive towards the compounds that affect K^+ channels. The sensitivity of cardiac cells towards neurotoxins suggests that when neurotoxins affect ion channels or pumps they will also most likely exhibit cardiotoxicity.

However, while beating is a more specific endpoint than cytotoxicity, the process behind the differentiation of embryonic stem cells into cardiomyocytes is time consuming and labour intensive, hampering the implementation of such assay for screening purposes. Nevertheless, EC_{50} values obtained with neuro-2a cells were in the same order of magnitude as that of the cardiomyocyte data, which are based on a more specific endpoint than cytotoxicity, and therefore seem to validate the cytotoxicity endpoint used in the neuro-2a mouse neuroblastoma assay. In

addition, a striking difference in sensitivity was observed for STX and TTX, for which the stem cell-derived cardiomyocytes were not as sensitive as the neuroblastoma neuro-2a assay, as the EC_{50} values for STX and TTX were almost three orders of magnitude lower in the neuro-2a assay. Sensitivity in the low nanomolar range, as obtained in the neuro-2a assay, is required to detect these marine biotoxins in contaminated samples. Beating cardiomyocytes might also not be sensitive to neurotoxins with modes of action other than affecting ion channels or pumps as for example binding to specific neuronal receptors.

In summary, this is the first study proposing a cardiac model for neurotoxicity testing. Despite its limitations, the assessment of the beating arrest in murine embryonic stem cell-derived cardiomyocytes represents an interesting tool for the screening of compounds for their neurotoxic properties. Murine stem cell-derived cardiomyocytes provide a sensitive model for the detection of specific neurotoxins and the neuroblastoma neuro-2a assay appears to be a more promising cell-based assay for the screening of marine biotoxins.

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