Considerations and Pitfalls in Selecting the Drug Vehicles for Evaluation of New Drug Candidates: Focus on *in vivo* Pharmaco-Toxicological Assays Based on the Rotarod Performance Test

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Received, December 8, 2017; Revised, March 8, 2018; Accepted, March 9, 2017; Published, March 13, 2018.

ABSTRACT - Purpose - During the discovery and development of new drugs, compounds with low aqueous solubility pose special challenges in their pharmacological evaluation and, therefore, the selection of appropriate vehicles to administer the compounds of interest is determinant for the quality of the results generated during the *in vivo* non-clinical studies. This work aimed to evaluate the motor deficit (as a surrogate of neurotoxicity) of several administration/delivery vehicles through the rotarod performance test. **Methods** - Trained male CD-1 mice were intraperitoneally administered with the following vehicles: dimethyl sulfoxide (DMSO), aqueous sodium chloride (NaCl) 0.9%, aqueous carboxymethylcellulose (CMC) 0.5%, polyethylene glycol (PEG)-400, propylene glycol (PG), and solutions of these vehicles containing 5% and 10% DMSO. **Results** - It was observed that the aqueous vehicles (NaCl 0.9% and CMC 0.5%) did not affect the performance of the animals on the rod. On the other hand, a vehicle consisting solely of DMSO led to significant motor impairment and only a small improvement was recorded over time. Additionally, a strong neuromotor toxicity was observed in the early evaluation points of the experiment using vehicles constituted by PG and PEG-400 or by mixtures of PG/DMSO (5% and 10%) and PEG-400/DMSO (5% and 10%). **Conclusion** - This study provides useful data about the neurotoxicity inherent to several vehicles frequently used in non-clinical pharmaco-toxicological assays, aiming to draw especial attention to the need of a careful selection of drug vehicles in order to avoid the impact of such confounding variables on the accuracy of the results and in decision-making processes.

INTRODUCTION

In the discovery and development of new drug candidates, the solubility of the test compounds is one of the physicochemical properties that must be considered and assessed since the early stages of drug research (1). Indeed, nowadays, it is widely accepted by the scientific community that the solubility of the drug compounds, especially its aqueous solubility, is a major indicator for the drug dissolution in physiological fluids, which is the limiting step for drug absorption and consequently to achieve the pharmacological activity (2,3). In fact, even for first *in vivo* preclinical screening studies, a suitable formulation strategy is required in order to enable an appropriate administration of the test compounds with acceptable tolerability and maintaining the stability for a sufficient period of time with no adverse effects in animal tests that could be attributed to the delivery vehicles (4,5). Particularly, when the compound of interest is developed to act in the central nervous system (CNS), its solubility is a very relevant challenge because, usually, is necessary a considerable degree of lipophilicity to cross the blood-brain barrier.

In this context, whenever possible, the choice of the delivery/administration vehicle falls in isotonic physiological saline solutions, which are considered innocuous. However, commonly, the test compounds are not soluble in this type of aqueous solvents due to their intrinsic lipophilicity and other options have to be considered. Dimethyl sulfoxide (DMSO), a polar organic solvent, often emerges as a relevant alternative, and many studies about its pharmacological and toxicological effects have been carried out over the years (6–8).

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Indeed, DMSO has been frequently included in different percentages in administration vehicles of compounds tested in whole-animal assays (9–11). Other examples of delivery vehicles widely used by the pharmaceutical industry in different formulations are propylene glycol (PG) (12) and polyethylene glycol (PEG) (13,14), which have the advantages of being soluble in polar and non-polar solvents and are quite inexpensive. In addition, they have been considered non-toxic (15,16). Furthermore, carboxymethylcellulose (CMC) is one of the most commonly used biopolymers in biomedical applications because it is also considered environmentally friendly and non-toxic (17–19).

The preclinical assessment of the "minimal neurological deficit" in rodents (mice and rats) is an essential task in primary and secondary pharmacological screening either in the early stages of drug development of new CNS-active drugs to screen out less promising compounds (20–24) or during the safety evaluation of peripheral-acting drugs in order to investigate adverse/toxic effects that could cause impairments later (25–27). Overall, the rotarod performance test has been widely used to indirectly assess the minimal neurological deficit in rodents induced by test compounds through the evaluation of the impairment of functions as balance and/or motor coordination. This behavioral assay has gained increasing importance in the discovery and development programs of new drug candidates as it is very simple to perform and allows the evaluation of a large set of compounds. Furthermore, the rotarod performance test is a versatile whole-animal assay that can be used for the assessment of any new molecular entity, regardless of its therapeutic area (28).

Over the last years our research group has been focused on the development of new antiepileptic drug candidates (29–34) and the selection of the administration/delivery vehicle to be employed for solubilization/suspension of test compounds continues to be a challenge in order to appropriately conduct pharmacokinetics and/or pharmaco-toxicological experiments in *in vivo* conditions. Hence, an analysis of the literature concerning the solvents and/or mixtures of solvents that are used to evaluate potential anticonvulsant compounds in *gold standard* assays of efficacy (maximal electroshock seizure test and subcutaneous pentylenetetrazole assay) and toxicity (rotarod test) (11,13,35,36) revealed that the impact of the administration vehicles on the obtained results has not been clearly evaluated and discussed. Although the influence of the delivery vehicle may be negligible in many pharmaco-toxicological assays, we suspected that this might not be the case in more sensitive behavioral assays, such as the rotarod performance test. Thus, the aim of the present study was to assess the minimal neuromotor impairment (neurotoxicity) induced by a set of the most common vehicles and their mixtures using the rotarod performance test.

MATERIAL AND METHODS

Chemicals and reagents

DMSO, CMC sodium and PG were obtained from Sigma (St. Louis, MO, USA). Sodium chloride (NaCl) 0.9% was obtained from B. Braun (Bethlehem, PA, USA). PEG-400 was obtained from Merck Schuchardt (Hohenbrunn, Germany).

Animals

Adult male CD-1 mice, aged between 6-7 weeks, were obtained from local certified animal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal). Four mice per cage were housed under controlled environmental conditions [12 h light/dark cycle (lights on at 8:00 AM) at 20 ± 2 ºC and relative humidity 50 ± 5%] with free access to tap water and standard rodent diet (4RF21, Mucedola, Italy). All experimental and care procedures were conducted in accordance with the European Directive (2010/63/EU) regarding the protection of laboratory animals used for scientific purposes.

Administration/delivery vehicles

Mice were intraperitoneally injected with each delivery/administration vehicle (10 µL/g of body weight). The vehicles assessed included the individual solvents (DMSO, NaCl 0.9%, CMC 0.5%, PEG-400 and PG) and also solutions of NaCl 0.9%, CMC 0.5%, PEG-400 and PG containing 5% and 10% DMSO.

Minimal motor impairment (rotarod) test

Minimal motor impairment was evaluated in mice by standard rotarod performance test as previously reported (37). Mice were previously trained to balance on an accelerating rotarod apparatus (rod diameter: 3 cm) that rotated at a constant speed of 10 revolutions per minute (Rota-rod, Ugo Basile, Varese, Italy). During the training sessions, the animals were placed on the rotating rod at least three consecutive trials for 90 s. On the day of the test, trained mice were injected with each delivery/administration vehicle and the motor/neurological toxicity was indicated by the inability of the animal to maintain equilibration on

the rod for at least 60 s (primary endpoint). The mice were placed on the rod at predefined time-points (0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h and 4 h) after the administration of each vehicle and fall off time was recorded (n = 4 per group). In this assay the number of animals that performed the test with success (primary endpoint) was recorded and, additionally, the number of seconds that each animal remained on the rod was also registered (secondary endpoint).

STATISTICAL ANALYSIS

Data were reported as the mean ± standard error of the mean. Comparison among groups was analyzed by using the one-way ANOVA with the *post hoc* Dunnett's multiple comparison test to judge significance of the observed effects. Differences were considered to be statistically significant for a p-value lower than 0.05 ($p < 0.05$).

RESULTS AND DISCUSSION

In this study, different administration vehicles were evaluated, which were chosen based on their potential usefulness for the solubilization/suspension and delivery of drug candidates in the first steps of non-clinical *in vivo* pharmaco-toxicological assays. The evaluation of the neurotoxic effects of the test set of administration vehicles was performed by means of the rotarod assay. This experimental evaluation has proved to have a remarkable value in the screening of potential side effects of drugs or drug candidates in the CNS, which are manifested on the balance and motor coordination required to successfully achieve the primary endpoint of the assay. In this comparative study we evaluated not only the potential neurotoxicity of the vehicles themselves (NaCl 0.9%, DMSO, PEG-400, PG and CMC 0.5%) but also solutions of these vehicles with different percentages of DMSO (5% and 10%).

Interestingly, it was observed that all vehicles containing NaCl 0.9% and CMC 0.5% with or without DMSO did not produce any motor impairment (Table 1). Probably this occurred due to the fact that these vehicles consisted in aqueous solvents and, therefore, they seem to be the safest option whenever they can be considered. Particularly, the vehicles containing CMC 0.5% with a small percentage of DMSO may be a good option to administer compounds that show poor solubility in NaCl 0.9% solutions. Indeed, several strategies have been developed to improve the solubility of compounds in this type of vehicles during the optimization phase of drug discovery and development. These include *in silico* methodologies to predict drug solubility, chemical modifications involving the preparation of prodrugs or the introduction of adequate functional groups, use of co-solvents and developing formulations to increase solubility and dissolution rate (38–40).

Regarding the discovery and development of new drug candidates, DMSO has become the solvent of choice to dissolve potential neuroprotective and neurotoxic hydrophobic substances used in pharmaco-toxicological research (41,42) that generally do not solubilize in aqueous vehicles. Some DMSO advantages include its capacity to dissolve compounds with a wide range of chemical properties, low volatility, miscibility with water, relatively low toxicity to both tissue culture and technicians, and limited deleterious effects at low concentration upon bioassays (43,44). In addition, it has been suggested that DMSO can be safely used, being generally well-tolerated by the experimental animals (45,46) and itself may still have benefits to specific disorders (47,48). However, there are also several case reports revealing severe neurotoxicity associated with DMSO, indicating the importance of its careful use in certain circumstances, including as administration vehicle, to avoid confounding factors that can bias the study results and lead to seriously erroneous conclusions (41,49,50). Because of these contradictions and the established importance of DMSO as drug vehicle, it was our interest to evaluate its neurological toxicity in *in vivo* non-clinical studies using the rotarod performance test. In fact, it was verified in the present study that the administration of a vehicle (10 µL/g) consisting of 100% DMSO originates motor impairment (a surrogate of neurotoxicity or minimal neurological deficit) on the rotarod assay, with animals falling from the rod at all post-dose time-points considered in the study (Table 1). In addition, it was observed that, at the first time-points, the animals receiving vehicles (10 µL/g) containing PEG-400 showed a notable toxicity, but at 4 h after the intraperitoneal injection, 100% of the animals successfully reached the primary endpoint of the assay. On the other hand, until 2.5 h after the injection of the vehicles containing PG, 75-100% of the mice showed inability to maintain equilibration on the rod for at least 60 s (primary endpoint). Moreover, considering the evaluation data for PG, it was verified that the animals receiving this delivery vehicle containing 10% of DMSO recovered the motor coordination faster than the animals

receiving only PG or PG with 5% of DMSO (neurotoxicity in 25% *versus* 75-100% of the animals at 4 h).

Besides the number of animals that successfully achieved the primary endpoint of the rotarod performance test (maintaining equilibrium on the rod for at least 60 s), it was also recorded the exact time in seconds (secondary endpoint) that each animal remained on the rod during the time set for the test. The results are illustrated in Figure 1. As can be seen, at the first time-points of the study it is possible to notice that the toxicity triggered by pure DMSO was lower than the observed with PEG-400 alone (1 h) and with vehicles containing PG.

Concerning the reasons explaining the DMSO-induced neurotoxicity, some evidence suggests that its mechanisms of toxicity can involve neurophysiological and pathological changes including myelin disruption and uncompacted myelin lamella (49). In addition, DMSO could also be able to produce widespread, dose-dependent neurodegeneration in the developing mouse brain at several ages and this toxicity probably results from a direct cellular effect. Furthermore, the DMSO-induced apoptosis might produce significant learning and memory deficits (50). Additional evidence has indicated that glutamate receptors could be involved in the neurotoxicity originated by DMSO (48); however, its exact mechanisms of toxicity remains unknown. Moreover, these studies just had focus on chronic neurotoxicity exhibited by DMSO and they probably could not explain the acute toxicity observed in our research.

In the present study it was also observed that all PG solutions possibly reached the brain rapidly because a strong neuromotor impairment in the rotarod performance test was noted. Particularly, the vehicles consisting of PG (100%) and PG/DMSO (95%/5%, *v/v*) in all steps of the study led to higher neuromotor impairment than the observed with 100% DMSO. On the other hand, although the PG/DMSO (90%/10%, *v/v*) solution originated evident neuromotor deficit in the early steps of the study, the recovery of the animals was faster than those receiving DMSO and the other vehicles containing PG. As demonstrated for DMSO, PG has also been suggested to produce apoptotic neurodegeneration in a dose-dependent manner. Furthermore, the observed damage was dependent on age at the time of exposure and probably PG does not produce damage through GABA receptors. It is still unknown whether apoptosis results in long-term cognitive and behavioral abnormalities (51).

After the administration of PEG-400–containing vehicles, interestingly, it was verified that with the increase of the DMSO percentage in the vehicle, the neuromotor toxicity seems to be reduced. In fact, 3 hours after the administration of PEG-400 with the highest percentage of DMSO (10%) all the animals performed the test without any evident neuromotor deficit. Actually, DMSO was reported as having anti-nociceptive and anti-inflammatory effects in male CD-1 mice when given orally (10 mL/kg) or by intracerebroventricular route (5 µL/mouse) (52), which could be a possible explanation for our results.

Regarding the PG-containing vehicles, another possible explanation for the neuromotor toxicity observed could be associated with the hyperosmolality effects and increase of the anion

Table 1. Time-course of minimal neurological impairment (neurotoxicity) of vehicles administered intraperitoneally to mice in the rotarod performance test (number of animals exhibiting neurological impairment/number of animals tested).

Vehicle	0.5 h	1 h	1.5 h	2 h	2.5 h	3 h	3.5 h	4 h
DMSO	4/4	4/4	4/4	4/4	4/4	3/4	3/4	3/4
NaCl 0.9%	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
NaCl 0.9%/DMSO (95%/5%, *v/v*)	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
NaCl 0.9%/DMSO (90%/10%, *v/v*)	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
PEG-400	4/4	4/4	4/4	3/4	2/4	1/4	1/4	0/4
PEG-400/DMSO (95%/5%, *v/v*)	4/4	3/4	3/4	3/4	1/4	0/4	0/4	0/4
PEG-400/DMSO (90%/10%, *v/v*)	4/4	3/4	2/4	2/4	2/4	0/4	0/4	0/4
PG	4/4	4/4	4/4	4/4	4/4	4/4	4/4	3/4
PG/DMSO (95%/5%, *v/v*)	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
PG/DMSO (90%/10%, *v/v*)	4/4	4/4	3/4	3/4	3/4	2/4	1/4	1/4
CMC 0.5%	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
CMC 0.5%/DMSO (95%/5%, *v/v*)	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
CMC 0.5%/DMSO (90%/10%, *v/v*)	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4

CMC, carboxymethylcellulose; DMSO, dimethyl sulfoxide; NaCl, sodium chloride; PEG-400, polyethylene glycol-400; PG, propylene glycol.

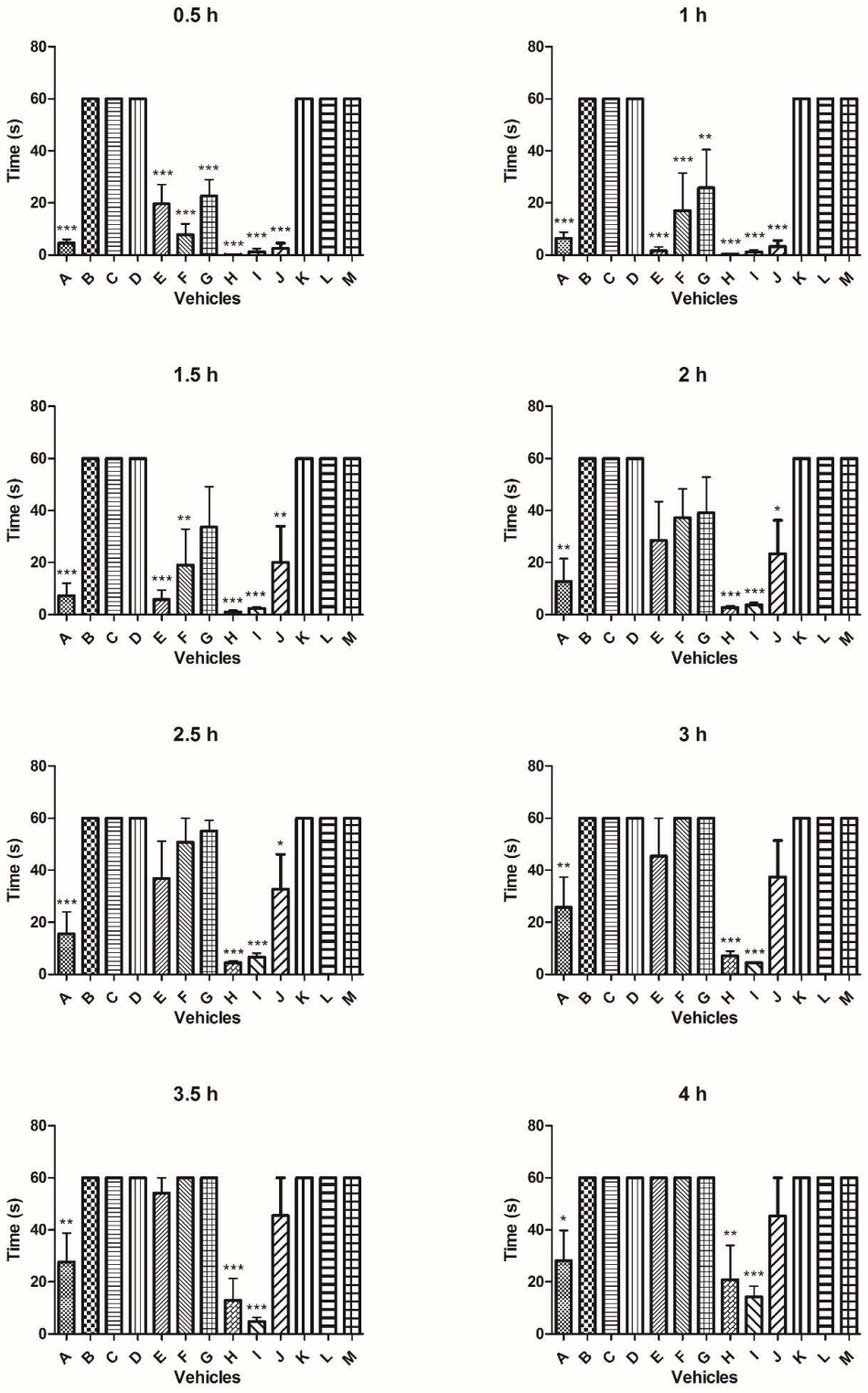

Figure 1 – Effects induced by commonly used drug vehicles on the time (in seconds) that intraperitoneally administered mice ($n = 4$) remained on the rod during the rotarod performance test. The test was performed at several predefined post-dose time-points (0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h and 4 h). **A** – Dimethyl sulfoxide (DMSO), **B** – Aqueous sodium chloride (NaCl) 0.9%, **C** – NaCl 0.9%/DMSO (95%/5%, *v/v*), **D** – NaCl 0.9%/DMSO (90%/10%, *v/v*), **E** – Polyethylene glycol (PEG)-400, **F** – PEG-400/DMSO (95%/5%, *v/v*), **G** – PEG-400/DMSO (90%/10%, *v/v*), **H** – Propylene glycol (PG), **I** – PG/DMSO (95%/5%, *v/v*), **J** – PG/DMSO (90%/10%, *v/v*), **K** – Aqueous carboxymethylcellulose (CMC) 0.5%, **L** – CMC 0.5%/DMSO (95%/5%, *v/v*), **M** – CMC 0.5%/DMSO (90%/10%, *v/v*). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control group (B).

gap metabolic acidosis (due to lactic acidosis) that was observed in humans. For instance, after an injection of lorazepam was observed that PG had a much greater contribution than PEG for the hyperosmolar metabolic acidosis (53). This information can be useful to understand the differences between PG and PEG-400 in our results.

Moreover, it should be highlighted that after the injections of PEG-400, PG and DMSO (100%) hypoactivity and immobility was noticed in the animals, which was consequently expressed in the performance on the rod. These results were in accordance with the observations of a previous study which analyzed several solvents administered intravenously in female CD-1 mice, which aimed to understand the tolerability and recommended solvent dose limits for pharmacokinetic studies (54).

Independently of the causes underlying the neuromotor toxicity observed with these delivery vehicles, this study shows that their use to evaluate the neurotoxicity of new drug candidates through the rotarod assay can be debatable. In fact, during the revision of literature it was frequently found the usage of PEG-400 (100%) (13,36,55–58) and even DMSO (100%) (11,42,59,60) as vehicles to assess the neurotoxicity of new antiepileptic drug candidates administered intraperitoneally, using the rotarod test. In these studies, the time-points for the evaluation of the motor impairment were coincident with two of the points of our study (0.5 and/or 4 h). In fact, the findings herein reported call into question the results obtained in the referred studies whenever the vehicles used have themselves a relevant neuromotor toxicity. Hence, it is clear that it is always necessary to evaluate the vehicles alone before the experiments with the compounds of interest in order to avoid possible confounding variables that influence the validity of the results.

CONCLUSION

The solubility of new chemical entities is one of the crucial parameters that has to be taken into account since the earliest steps of programs of discovery and development of new drug candidates. In fact, compounds with insufficient solubility not only can be problematic in the interpretation of results, but also can lead to additional costs. Thus, the selection of appropriate vehicles to incorporate the compounds of interest is of paramount importance to have confidence in the experimental results obtained. In this study the motor impairment (a surrogate of neurotoxicity or minimal neurological deficit) provoked by several vehicles was assessed through the rotarod performance test. As expected, all the aqueous vehicles (NaCl 0.9% and CMC 0.5% with and without DMSO) showed to be safe in this test. However, other frequently employed vehicles, such as DMSO, PEG-400 and PG induced marked neuromotor deficit in mice. This means that relevant misrepresentations of the experimental results could occur by using these vehicles to administer poorly soluble compounds to be evaluated in the rotarod performance test. In this way, this study brings further important data on the neuromotor deficit/neurotoxicity of some commonly used administration/delivery vehicles. Accordingly, this work appeals to a special attention in the selection of drug vehicles, in order to avoid the impact of such confounding variables on the accuracy of the results and in subsequent decision-making processes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The authors are grateful to Fundação para a Ciência e a Tecnologia (Lisbon, Portugal) for the PhD fellowship of Mariana Matias (SFRH/BD/85279/2012), involving the POPH–QREN, which is co-funded by FSE and MEC. The authors also acknowledge the support provided by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalisation in Axis I - Strengthening research, technological development and innovation (Project No. 007491) and National Funds by FCT - Foundation for Science and Technology (Project UID/Multi/00709/2013).

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