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Correct assessment of new compounds using *in vivo* screening models can reduce false positives

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During early drug discovery the initial *in vivo* efficacy testing is often performed in rodent models optimized to screen and select lead compounds rapidly, before progressing them to *in vivo* models that reflect the human form of the disease more closely. The way such models are frequently run can risk overestimating the efficacy of new compounds when using pre- and co-administration, as shown in three examples from different central nervous system research areas. This is undesirable for reasons ranging from good decision-making, cost efficiency and time management to the ethics of animal use. Abandoning the use of pre-treatment, monitoring crucial physiological parameters in (satellite) animals and systematically applying simple pharmacokinetic–pharmacodynamic analysis could reduce the number of false positive results.

During early drug discovery *in vivo* models that are optimized to screen lead compounds rapidly for beneficial drug effects, rather than reflecting the human form of the disease as closely as possible, are often used. These screening models are first in line when a new entity is progressed to *in vivo* studies. A positive result in this model is usually followed up by experiments in a more stringent disease-like model, if such a model exists. Most of these initial screening models are in rodents and are based on similar principles: animals are treated with a pharmacologically active compound, often to induce some sort of lesion, or subjected to a simple surgical procedure that induces an acute and specific response or injury, which is subsequently treated with the experimental compounds of interest. Within the central nervous system (CNS) research area, applications of these types of models are plentiful: formalin-induced pain, scopolamine-induced impaired learning and memory, MPTP-induced Parkinsonian syndrome, pentatetrazole-induced seizures, kainate-induced excitotoxic lesions, among others.

To test new compounds using *in vivo* screening models, many academic and industrial research groups make use of a co-administration or pre-treatment regimen (i.e. the experimental drug is administered simultaneously with or in advance of the agent inducing the treatable response or injury) and measure the efficacy of treatment at one discrete time. There are basically two potential problems connected with this treatment protocol. First, there is a possibility of the new compound of interest directly interacting with the development of the treatable response or injury; such an interaction results in an overestimate of the efficacy or potency of the new compound. Second, the relationship between the concentrations of the new compound and the effects it generates, often referred to as the pharmacokinetic–pharmacodynamic (PKPD) relationship, cannot be studied. Often no (or only sparse) pharmacokinetic information is collected and the time profile of the effect is not, or cannot, be properly captured. An additional problem is the unstable baseline behaviour of some of these models (i.e. the induced response or injury in the control animals also changes continuously over time). This makes it challenging to judge whether adequate exposure was reached in the brain over a long enough period, and whether this makes sense in view of the observed efficacy of the compound. These issues sometimes result in new lead compounds going forward as promising, whereas they may not prove to be all that good after more detailed and stringent studies. This is highly undesirable for reasons ranging from good decision-making, cost efficiency and time management to the ethics of animal use. A great deal of time and effort is needed before a false positive result is rectified and, even worse, the synthesis programme may also be sent in the wrong direction, causing additional delays.

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Here, we will summarize a few examples from the research into CNS diseases that illustrate the risk of using pre-treatment or co-administration regimens. The order in which these examples are presented reflects the likelihood of their occurring in early drug discovery. In the first example the observed efficacy (at least partly) turned out to be caused by an interaction with the development of the induced injury rather than because of its target receptor. The second example shows that an entirely different mechanism of action was responsible for the observed efficacy as a consequence of pre-treatment of the experimental drug. The third example shows that the compound of interest unexpectedly interacted with the anaesthetic used in the experiments, which explained its efficacy. Finally, some suggestions are presented that reduce the chance of overoptimistic results from *in vivo* screening models.

Example 1: 7-NI protects against MPTP-induced neurotoxicity

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes degeneration of dopaminergic neurons in the substantia nigra in rodents, primates and humans, which replicates many of the symptoms of Parkinson's disease. To exert its neurotoxic effect, MPTP needs to be metabolized by monoamine oxidase B (MAOB) to form 1-methyl-4-phenylpryridinium (MPP^+). MPP^+ accumulates selectively in the dopaminergic nerve terminals and initiates several detrimental processes that ultimately kill the neurons [1,2].

Treating rodents and baboons with the neuronal nitric oxide synthase (nNOS) inhibitor 7-nitroindazole (7-NI) protected against MPTP poisoning [3–6]. These neuroprotective effects were reported to be caused by decreased nitric oxide production. Nitric oxide can react with MPP^+-produced oxide radicals to form the highly reactive peroxynitrite species, which is thought to contribute to the demise of the dopaminergic neurons. In mice, pre-treatment with 50 mg/kg of 7-NI completely prevented MPTP-induced striatal dopamine depletion and niagral cell loss [3,4,6,7]. The striking effects of 7-NI resulting in complete neuroprotection suggested a primary involvement of nNOS in the MPTP-induced pathology. Only 50% protection was observed with *S*-methylthiocitrulline, another selective nNOS inhibitor [8], and no protection was found with the non-selective NOS inhibitor L-NAME given before MPTP [6,9]. Also, vulnerability to MPTP toxicity was decreased in nNOS knockout mice, but not completely abolished [4]. These findings raised the question as to why 7-NI showed superior efficacy compared to the other NOS inhibitors in the MPTP mouse model. Studies in which the toxic species MPP^+ was carefully monitored in the brain revealed that the concentrations of MPP^+ were significantly reduced in the 7-NI pre-treated animals following MPTP injection by up to approximately 50%, depending on the administered dose and route of MPTP and the time of measurement [10–12], but see also Reference [4]. Besides being an nNOS inhibitor, 7-NI also competitively inhibited MAOB both *in vitro* and *in vivo*, thereby concentration-dependently attenuating the conversion from MPTP to MPP^+ and modifying the degree of MPP^+-induced injury in the brain [10,11,13]. Unfortunately, a more complete pharmacokinetic profile to establish the detailed relationship between central 7-NI concentrations and the rate of conversion of MPTP to MPP^+ is lacking. Other known MAOB inhibitors such as pargyline and deprenyl show similar neuroprotection against MPTP toxicity [7,14,15] to that given by 7-NI. Recently, a similar case was presented in which the neuroprotective properties of pioglitazone (PPARγ agonist) in this model also appeared to be as a result of MAOB inhibition rather than the result of modulating glial cell activity [16].

In summary, 7-NI not only protects dopaminergic neurons by inhibiting nNOS but also directly influences the development of the neuropathology itself via MAOB inhibition, which leads to an overestimate of the protective effect of this particular inhibitor. The lower efficacy against MPTP-mediated neurotoxicity by other NOS inhibitors and knockout animals clearly hinted to a problem for 7-NI specifically. The interaction would be of much less importance if 7-NI were administered after MPTP as opposed to the pre-treatment protocol, as was done in these studies. Indeed, the administration of 50 mg/kg of 7-NI 3 h after MPTP did not provide any significant protection against MPTP-induced dopamine depletion [6].

Example 2: Adenosine A_1 receptor agonists protect against sarin poisoning

The nerve-gas agent sarin causes a strong accumulation of acetylcholine in the synaptic cleft by inhibiting acetylcholinesterase (AChE), which is responsible for the metabolism of acetylcholine. This leads to an excessive accumulation of acetylcholine that might be the initiator of the sarin-induced seizure activity and associated brain damage [17–19].

Selective adenosine A_1 receptor agonists inhibit the release of acetylcholine and excitatory amino acids [20,21]. Furthermore, it has been shown that they also possess anti-convulsive and neuroprotective properties [22]. Stimulation of these adenosine A_1 receptors in the brain could therefore be a new and general strategy to counteract specifically the CNS effects of nerve gas poisoning [17].

Intramuscular treatment 2 min before or 1 min after sarin poisoning of rats with 1–2 mg/kg of the selective adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA, K_i = 5.9 nM [23]) dose-dependently reduced centrally mediated symptoms, such as convulsive activity and respiratory failure, seen following a lethal subcutaneous sarin injection, leading to a remarkable survival rate of 100%. These impressive improvements were associated with the prevention of accumulated extracellular acetylcholine in the striatum [24]. From these results it was initially concluded that CPA effectively protects against sarin intoxication via adenosine A_1 receptors located in the brain. 2dCPA (2′-deoxy-N^6-cyclopentyladenosine, K_i = 1.9 μM [23]), a close analogue of CPA, however, failed to protect adequately against sarin poisoning in sharp contrast to CPA [25]. Rats suffered from all the monitored symptoms, the survival rate dropped to below 30% and excessive extracellular acetylcholine concentrations were found in the striatal tissue. Local administration of CPA and 2dCPA in the brain showed that both these compounds reduced acetylcholine release concentration-dependently to 50% of baseline values, albeit with different potencies, 0.24 μM and 96 μM respectively [26]. Moreover, both agonists completely arrested sarin-induced epileptiform activity in the guinea-pig hippocampal slice model, with potencies of 4.8 nM and 113 nM [27]. In the light of the similar efficacy of both adenosine agonists in these *in vitro* and *in situ* experiments, it

was difficult to explain why CPA successfully protected against sarin intoxication *in vivo*, whereas 2dCPA did not.

After a search for the mechanism behind the protective properties of CPA against sarin poisoning, it turned out to be mediated via the cardiac A_1 receptors [28,29]. The profound bradycardia (a fall of 208 bpm) and hypotension (a fall of 60 mmHg) following CPA administration affected the delivery of sarin to the brain, thereby indirectly protecting against sarin poisoning. Detailed studies of the kinetics by which sarin inactivated AChE revealed that they were affected by CPA, depending on when (pre- or post-dosing) CPA was administered relative to sarin. Pre-treatment of rats with CPA delayed the irreversible inhibition of AChE in the blood and spared a fraction of AChE in the brain. It was estimated through PKPD modelling that the fraction of the dosed sarin reaching the AChE population in the brain was reduced by 87% as a consequence of CPA pre-treatment and thus cardiac side effects. Since sarin has a very short half-life in a biological system, it could not reach the brain in time and only reacted with other proteins in the tissues outside the brain [30]. Additional evidence in support of this was the report that a peripherally restricted and selective A_1 receptor antagonist, which blocked adenosine A_1 receptors located in the heart but not the brain, could abolish the protective effects of CPA [31]. Recent observations have also pointed to a prominent role for cardiac A_1 receptors in the depression of evoked field potentials in the hippocampus, which was previously assumed to be mediated by central adenosine A_1 receptors [32]. 2dCPA behaves as a partial agonist of the cardiac adenosine A_1 receptors, thereby reaching a maximum effect of only 30% relative to CPA, which explains why this compound failed to protect against sarin intoxication.

In summary, the studies described showed that CPA protects rats against sarin poisoning and prevents acetylcholine accumulation in the brain, which presumably explains the absence of lethal toxicity. In contrast to what was initially expected, however, the attenuation of acetylcholine was not mediated by adenosine A_1 receptors residing in the brain, but by the cardiac A_1 receptors, which appeared to be primarily responsible for the protection against sarin. This also implies that the impact of the cardiovascular effects would be of much less significance for a nerve gas agent with a significantly longer half-life than sarin. In such a case the cardiovascular effects would only delay the penetration into the brain rather than prevent it. Indeed, CPA was totally ineffective against VX or parathion [24].

Example 3: MK-801 protects against neuronal damage after ischaemia

A myriad of mechanisms leading to brain injury after transient global ischaemia have been identified. They include the production of oxygen free radicals, release of various inflammatory mediators, release of calcium, failure of cellular energy metabolism and other mechanisms. Much evidence also points to the involvement of *N*-methyl-D-aspartate (NMDA) receptors, which are overstimulated by excessive, but transient, glutamate release following an ischaemic event [33–36]. This results in toxic concentrations of intracellular Ca^{2+}, which initiates deleterious excitotoxic mechanisms. A hallmark of transient global ischaemia is the selective and delayed death of the hippocampal CA1 neurons [34,37].

Bilateral carotid artery occlusion in the gerbil represents a simple and convenient model for testing new compounds in the treatment of transient global ischaemia. The treatment of ischaemic gerbils with 0.3–10 mg/kg of the non-competitive NMDA receptor antagonist dizocilpine (MK-801), before or shortly following the induction of transient global ischaemia, prevented hippocampal neuronal loss completely, or almost entirely, at four days after ischaemia [38,39]. From these studies it was concluded that the potent neuroprotective effects of MK-801 proved the central role of the NMDA receptor in the selective hippocampal neurodegeneration following global ischaemia. However, it was shown years later that MK-801 also causes hypothermia in combination with the anaesthetics used in these animals [40,41]. Temperature dropped by 3–7 °C in gerbils, depending on the dose of MK-801 administered. When adjustments were made for temperature fluctuations in the gerbils, no evidence of histological or behavioural protection by MK-801 was seen. Hypothermia by itself has been known to reduce or completely prevent ischaemic damage in various animals, probably owing to a decrease in energy demand [40,42,43]. In the majority, but not all, of more stringent rat ischaemia models, it was confirmed that MK-801 is not very successful in protecting against global ischaemia [44].

In summary, MK-801 shows robust neuroprotection in ischaemic gerbils, although these results are mainly explained by the hypothermia it causes during the development of the ischaemic damage rather than by antagonism of glutamate. This resulted in an overestimate of the efficacy of MK-801 in the treatment of global ischaemia.

Suggestions for avoiding these misinterpretations

These afore-mentioned examples clearly demonstrate some of the risks involved in starting treatment before or very shortly after the inducing agent or surgery producing the response or injury to protect against. The efficacy of a new compound or a group of compounds can be overestimated in an *in vivo* screening model, leading to overoptimistic expectations. This may lead to incorrect conclusions and decisions on the progression of specific compounds, which is undesirable from a cost, time and ethical point of view. Some relatively simple measures, however, can be taken to reduce the possibility of these false positive results occurring.

Experimental design

First of all, whenever possible, experimental compounds should be administered to animals a reasonable time after the treatable response or injury is initiated to minimize the risk of interactions with the development of the response or injury itself. This implies that the often unstable baseline response needs to be characterized over time before the administration of any treatment. There are scenarios imaginable in which pre-treatment with an experimental drug is a necessity owing to its specific mechanism of action or because a study otherwise becomes nearly impossible to conduct. In these cases it is important to identify and control for crucial off-target interaction possibilities in the model, such as MAOB inhibition in the first example. In the MK-801 case, the compound had the desired effect because of an unexpected interaction with the used anaesthetic in the experiment. Alternatively to avoiding pre-treatment, this false positive could possibly be avoided by modifying the experimental conditions, for instance by using a

different anaesthetic. It takes a great deal of effort and insight, however, to discover such an unexpected interaction and this is easily overlooked when screening large numbers of new compounds. Therefore, abandoning pre-treatment might be a more secure way to circumvent this type of drug–drug interaction. Within the models described here, there are examples of compound treatments that were protective after the injury or toxin was administered, although only a few exist for the MPTP mouse model. For instance, modifinil is partially protective when given up to 3 h after MPTP in mice [45], galantamine protected hippocampal cells when administered up to 3 h after global ischaemia in gerbils [37,46], and caramiphen and benactyzine attenuated brain damage when given 10 min after the first signs of sarin-evoked convulsions [47].

Secondly, crucial physiological parameters that have major impact on the development of the treatable injury should also be monitored in the presence of pharmacological treatment. This is often fairly easy and may be done in satellite animals if it is contraindicated in the animals that are in the efficacy study (when dealing with a behavioural endpoint, for instance). The recommendations with regard to preclinical models used to develop new therapeutic approaches given by the STAIR (Stroke Therapy Academic Industry Roundtable) committee are of interest in this connection [48,49]. Among these are that drug administration before or shortly after the onset of ischaemia should be prevented and the relevant physiological parameters monitored. These recommendations follow the observation that only two thrombolytic therapy trials have demonstrated efficacy against stroke in humans, whereas a large number of other clinical trials have failed. A new study that examined all the available preclinical material for five drugs intended for stroke treatment (clomethiazole, gavestinel, lubeluzole, selfotel and tirilazad) showed that the start of treatment was 10 (−60 to 360) min (median (range)) [50] after the onset of ischaemia, again indicating that in many preclinical studies treatments are started before or immediately after induction of the treatable injury. Other research areas have adopted the initiative of STAIR, including the AD Neuroimaging Initiative Consortium (ADNE) [51] and the Treatment Units for Research on Neurocognition and Schizophrenia (TURNS), and are also pursuing increased quality of preclinical models and studies [52].

Rudimentary PKPD analysis

All data generated within a class of new compounds should be considered and understood. Occasionally, data are interpreted in an oversimplified manner [52] or even dismissed when a compound appears not to be active. Compounds showing a robust effect usually generate much larger momentum and energy than compounds failing to do so, whereas both may give an equal amount of information on the target of interest. In the examples of the protection of 7-NI against MPTP-induced neurotoxicity and CPA against sarin poisoning, it was not the efficacy of these compounds that raised a warning, but rather the analogues that should produce an effect via similar mechanisms but failed to do so. By establishing a rudimentary PKPD relationship for reference compounds, if any exist, or frontrunner compounds in such models, such errors may be circumvented. By a rudimentary PKPD relationship is meant that the relation between exposure and response is characterized and an attempt is made to understand possible dose and time dependencies. Although this PKPD approach does not necessarily prevent possible interactions between drugs and the *in vivo* models from occurring, discrepancies between efficacies or potencies of compounds are identified earlier. An additional advantage of establishing such correlations is that it might even uncover false negative results. Just as it is possible to overestimate the efficacy of an exploratory drug owing to interactions, it is possible that beneficial effects go unnoticed as a result of competing mechanisms or adverse effects that minimize the desired outcome.

So far during the early drug discovery phase, the application of PKPD modelling has been rather limited, if anything. Producing sufficient data and especially developing a mechanism-based PKPD model cost a lot of time [53,54], which is incompatible with the short deadlines of the drug discovery programme. Consequently, the focus should not necessarily be aimed at modelling such a relationship and the technicalities as such, but rather at applying the basic principles. Characterizing simple correlations between pharmacokinetics and pharmacodynamics is highly appropriate in early drug discovery. In essence, PKPD is nothing more than a rational way of treating data based on the primary idea that the concentration at the target site steers the observed response either in a direct or delayed manner. In principle, therefore, *in vitro* potency ratios should be conserved *in vivo* when taking into account differences in exposure/pharmacokinetics, plasma protein binding and species differences. Also effects do not necessarily have to be measured at C_{max} but rather at different exposure levels [53,54]. Measuring exposure in (satellite) animals is crucial and subsequent comparison of exposure and understanding the processes leading to efficacy (fast or slow processes that lead to rapid or slow onset of effect) can give valuable information when comparing various candidates/lead compounds. It is important to compare experiments (different dosing regimens, administration routes) for reference/lead compounds in order to evaluate and adjust hypotheses about the mechanism of action. Results from previous leads could be used to predict responses for new entities based on an assumed mechanism of action. Deviation from expectations will flag for other possible mechanisms involved. This makes close collaboration between *in vitro*, *in vivo* pharmacology and DMPK (drug metabolism and pharmacokinetics) a prerequisite.

Concluding remarks

Rapid *in vivo* screening models and the way they are run involve a risk of overestimating the efficacy of new compounds when using pre- and co-administration, as shown for three independent examples. In drug discovery, false positives resulting from target-related side effects of competing mechanisms on different targets are commonplace and should be expected. Such interactions could be predicted in some circumstances based on receptor distribution in various organs or by *in vitro* screening panels, in which the relative binding to a selection of receptors are characterized. Issues, however, such as tissue-specific efficacy, such as is the case with adenosine agonists complicate these predictions. Moreover, larger receptor binding screening panels – still far from complete – are often only run at the end of the lead optimization phase when the damage may already have been done. Preventing pre-treatment with new compounds, identifying and checking for crucial

physiological parameters in (satellite) animals and systematically applying simple PKPD principles are simple measures that could circumvent the risk of overestimating efficacy and increase awareness of inconsistent results in chemical series.

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