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Vehicles for Lipophilic Drugs: Implications for Experimental Design, Neuroprotection, and Drug Discovery

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Abstract: The delivery of some classes of drugs is challenging. Solubility, absorption, distribution, and duration of action may all be altered by combination with vehicle molecules. It has already been discovered that polyethylene glycol – which is used as a stabiliser in peptide drug formulations – has biological activity in its own right, including potential neuroprotective properties. In this article we review the evidence for confounding activity for four distinct compounds that have been used as solvents and/or carrier molecules for the delivery of lipophilic drugs under investigation for potential neuroprotective properties. We discuss the evidence that cyclodextrins, ethanol, dimethyl sulphoxide, and a castor oil derivative - Cremophor™ EL – have all been found to have mild to moderate neuroprotective effects. We argue that this has probably reduced the statistical power and increased the Type II error rates of neuroprotection experiments that have employed these vehicles, and suggest experimental design considerations to help correct the problem. However, we also note that the properties of these compounds may represent an opportunity for drug development, particularly for the newer compounds that have been subject to only limited experimental investigation.

Keywords: Carrier, Cyclodextrin, DMSO, Ethanol, Cremophor, Lipophilic, Neuroprotection, Vehicle.

INTRODUCTION – WHEN DRUG VEHICLES AND ADDITIVES ARE NEUROPROTECTIVE

It is not always the case that a drug can be delivered effectively in its base form in preclinical research, i.e., dissolved only in saline. Instead, various additives are often used to promote absorption, distribution, and appropriate duration of action. This is particularly the case for highly lipophilic drugs, which require the use of strong solvents in order for the drug to be miscible and therefore experimentally useful. However, these additives may have biological activity themselves. Sometimes this activity takes the form of an adverse effect, but sometimes the activity can cause – at least partially – the same outcome as the drug under investigation. The purpose of this article is to review a number of vehicles that have are commonly used for highly lipophilic drugs in studies of neuroprotection, and the evidence for their having neuroprotective properties themselves. It will be argued that this reduces the statistical power of experiments that have used these vehicles, and may have led to a number of Type II "false negative" results, such that some otherwise promising drugs may have been discarded. It will also be argued that when new vehicles are found to confound an experiment in this way, then this may represent an opportunity for drug development in its own right.

The neuroprotective properties of a common drug additive/vehicle can perhaps be seen most clearly with the example of polyethylene glycol (PEG), which is not used to dissolve lipophilic drugs, but as a protein stabilizer to reduce clearance and increase duration of action for protein-based drugs. Because of the general nature of its mechanism of action, PEG has many other uses in industry and medicine. It has now been known for several decades that PEG has neuroprotective properties [1, 2], including antioxidant [3, 4] and repair properties [5, 6]. In this article we will discuss several vehicles for lipophilic drugs – rather than peptides in the case of PEG – and review the evidence that some or all of them have mild to moderate neuroprotective properties.

ACTIVE VEHICLES FOR LIPOPHILIC DRUGS

Highly lipophilic drugs are difficult or impossible to dissolve in water based buffers without the use of a solvent. These solvents act by decreasing the surface tension of the water to allow non-polar molecules to form an emulsion [7]. In biology careful consideration must be used when deciding which solvent should be used in the vehicle and at what concentrations they should be used. Because of this, working with highly lipophilic drugs can be challenging in some fields of research. For example, cannabinoids tend to be extremely water insoluble, and preparing drugs for administration at a volume that allows for precise dose titration invariably involves the use of solvents or carrier molecules of some kind. In some kinds of research where extremely small volumes are required – for example, for intrathecal delivery – the amount of vehicle substance may become very large in comparison to the amount of base drug delivered, as the drug reaches the limit of solubility relative to the volume of vehicle that can be used. This is less of a problem where larger dosing volumes can be used, but can still potentially confound experimental results. In the sections below several drug vehicles that are commonly used

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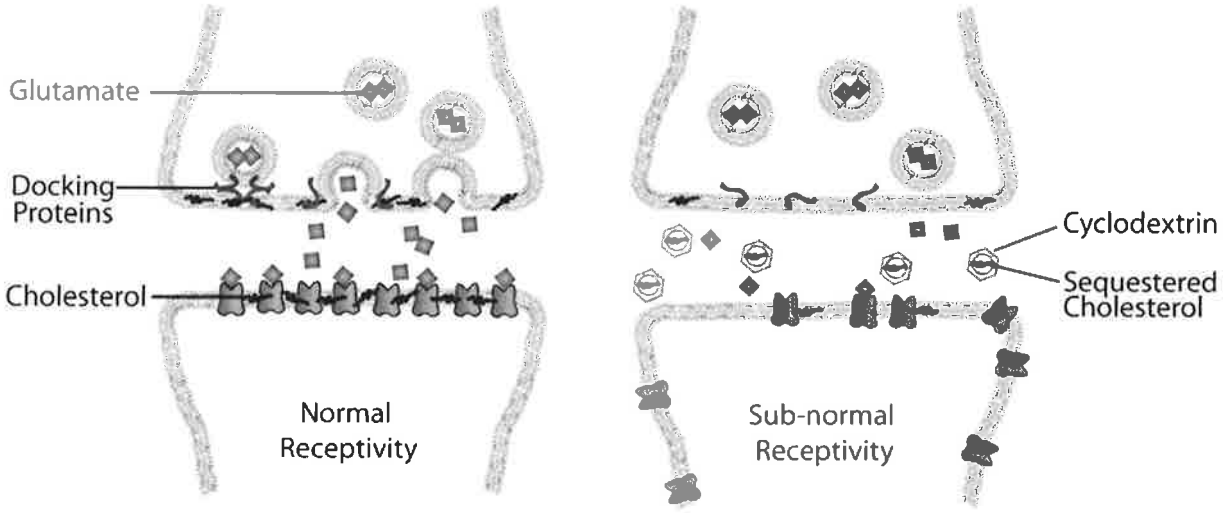

Fig. (1). Cholesterol is important for the function and density of proteins within the cellular membrane at the synapse. Docking proteins require cholesterol to function correctly and facilitate vesicle fusion with the cellular membrane and receptors require cholesterol to prevent receptor diffusion throughout the membrane and maintain receptor density at the synapse. Cyclodextrin sequesters cholesterol from the membrane causing reduced vesicle fusion and results in the diffusion of receptors throughout the cellular membrane.

in neuroprotection experiments are discussed with respect to the evidence that the vehicles themselves can have neuroprotective effects.

CYCLODEXTRINS

Cyclodextrins are cyclic oligosaccharides that have a hydrophilic surface and a lipophilic central. Lipophilic chemicals can associate with the core of cyclodextrin while in a water solution; this allows the lipophilic chemical to dissolve in water. Because of these properties, cyclodextrins can be used as carriers for lipophilic drugs [8]. However, cyclodextrins are also biological effector molecules in their own right. Some cyclodextrins alter lipid raft structure by removing cholesterol from cell membranes [9, 10]. Cholesterol is crucial in the formation of lipid rafts, which are microdomains within the cell membrane that have unique lipid and protein composition and hence a uniquely arranged and ordered structure. This property of lipid rafts is instrumental in the formation of synaptic densities, which contain high densities of receptors, such that alterations in cholesterol content of lipid rafts can alter receptor function [11]. Furthermore, cholesterol rich lipid rafts have been shown to be essential for vesicle fusion and exocytosis and it has been shown that removal of cholesterol from the membrane results in reduced exocytosis (Fig. **1**) [12, 13].

There is direct evidence that cyclodextrins can alter receptor function at synapses. Methyl-b-cyclodextrin (MB-CD)has been found to impair long-term potentiation (LTP) in rat hippocampi [14] and impair intracellular calcium influx [15, 16]. Frank *et al.* (2008) demonstrated that co-application of cholesterol with MB-CD prevents attenuation of LTP. However, in other experiments washout of MB-CD before LTP induction did not rescue LTP attenuation [14]. This suggests that MB-CD irreversibly induces disruption of lipid membranes and rafts, and potentially long duration of action. The disruption of lipid rafts by MB-CD alters signalling properties of N-methyl-D-aspartate (NMDA) receptors and 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic(AMPA) receptors, key receptor systems for the induction and expression of LTP respectively [17], but also critical for excitotoxicity, and both associated with cholesterol-rich membrane micro-domains [18, 19].

Alterations in excitatory neurotransmitter receptor function at synaptic densities should be expected to have effects on brain damage during and after cerebral ischemia. There are several types of evidence to support this hypothesis. First, several classes of cyclodextrin protect cultured cortical neurons against oxygen-glucose deprivation, and against N-methyl-D-aspartic acid (NMDA) and glutamate induced excitotoxicity, without affecting normal calcium signalling [20]. Methyl- β-cyclodextrin has also been found to protect hippocampal slices from the effects of anoxia [21]. One of the neuroprotective cyclodextrins investigated by Abulrob *et al.* [20], 2-hydroxypropyl-β-cyclodextrin (HP-CD), has also been extensively characterised in rats by Valenzano *et al.* [8]. The authors of that study found that intraperotineal injection of 10mL/kg 25% HP-CD did not cause any neurological impairment or other adverse effects, but Rivers *et al.* [22] found that not only could HP-CD lower the excitability of neurons in hippocampal *ex vivo* preparations, but was also moderately neuroprotective in a rat model of hypoxia-ischemia (HI). It is possible that this was a result of decrease excitation of the neurons as neurotransmitter exocytosis and receptivity was reduced, due to cholesterol sequestering by the HPCD and the subsequent breakdown of lipid

DIMETHYL SULFOXIDE (DMSO)

DMSO is an organosulfur that is used as an aprotic solvent for both polar and nonpolar compounds. Because it is miscible in water and various organic solvents it is commonly used as a drug solvent. DMSO is one of the most widely used solvents in medical research; particularly as a solvent/vehicle for lipophilic compounds. However, it has many effects on the body [7, 23]. DMSO has been shown to have antioxidant, anti-inflammatory and anticoagulatory effects, and through these mechanisms DMSO has been shown to be protective in many pathologies [7]. Di Giorgio

et al. [24] use a model of traumatic brain injury to show that DMSO can be neuroprotective in the infarction penumbra. DMSO treated animals had approximately 50% less neuronal death than the saline control group. Di Giorgio *et al.* (2008) used three 1ml/kg injections of 100% DMSO administered directly before the injury, 30min after and again 90min after the injury, totaling 3ml/kg of DMSO.

A possible mechanism of neuroprotection by DMSO was reported by Greiner *et al.* [25], who used *ex vivo* hippocampal slices to establish that DMSO lowered levels of hypoxia induced depolarization in a concentration dependent manner. This was reportedly due to the DMSO directly acting of ion channels, blocking ion transport across the cellular membrane and thus lowering the levels of depolarization. The study found that the concentration required to elicit a statistically significant response was 0.4% of the artificial cerebral spinal fluid applied to the hippocampal slice. An *in vivo* study of the neuroprotective effects of DMSO in the MCAO model in rats was performed by Shimizu *et al.* [26], who found that administration of 0.1ml of DMSO was protective. The study used the same dose volume for all rats which reportedly weighed between 275-300g. This corresponds to a minimum efficacious dose of 0.364 to 0.333ml/kg (the next dose of 0.10 to 0.11ml/kg was not efficacious). DMSO has also been shown to be anti-inflammatory through inhibition of nuclear transcription factor kappa-beta (NF-κB) signaling [7, 27]; mechanisms of neuroprotection may therefore include both an anti-inflammatory component and an anti-excitotoxicity component.

CREMOPHOR™ EL

Cremophor™ EL (C-EL) is a solvent in common use in medical research and is used for several chemotherapy drugs in the clinic [28]. It is formed by reacting ethylene oxide with castor oil to form polyethylene glycol ethers. As it is made from castor oil which is extracted from the bean of the *Ricinuscommunis* plant, it has a heterogeneous composition. Very little research has been done on the neuroprotective effects of C-EL in any model of brain damage. A thorough search of Pubmed™ and Web of Knowledge™ journal databases using many combinations of key words including the new trade name of C-EL, Kolliphor EL™ returned one research article with relevant data. The article reported upon the primary goals of testing the neuroprotective effects of cyclosporin A and tacrolimus (FK506) on a brain trauma model in different aged rats (P6 and P30) [29]. There were four treatment groups: brain trauma only, cyclosporine, tacrolimus, and drug vehicle only. The drug or vehicle administration occurred 20min after the injury and again 24h after. The injection volume was not stated. The authors found that the 0.25%(v/v) C-EL and 0.25% Ethanol(v/v) in saline vehicle had the lowest infarction volumes; cyclosporin A and tacrolimu were reported to abate the protective effects of the vehicle and the injury only animals had the largest infarction volumes. Without knowing the dose volume the total C-EL administered cannot be calculated. However, based on the concentration of C-EL used and commonly used injection volumes, it is likely that the total C-EL dose was very small. It is surprising that only one study has reported this effect and no more studies have been released since it was first published in 2007. This is particularly interesting considering the number of researchers that use C-EL as a vehicle.

Other studies have reported other side-effects that indicate that C-EL may have a pathological effect following a cerebral ischemic injury such as increasing the immune response to injury and peripheral axonal damage when applied topically [28, 30]. Although the effects of C-EL in cerebral ischemic injury have not be researched and little research has been done on other brain injuries, the neuroprotection reported by the Setkowixz and Guzik (2007) is worth discussing as it may lead to research that implicates C-EL as a future neuroprotectant.

ETHANOL

In the study by [29] discussed above, C-EL was combined with 0.25% ethanol, which itself may have neuroprotective properties. Ethanol is an excellent solvent with a low toxicity profile. However, it has of course well documented effects on the body, particularly the brain. Ethanol's primary effect on the brain is to enhance γ-Aminobutyric acid (GABA) signaling, which results in increased inhibition of neuronal firing [31]. This may be neuroprotective by reducing the level of excitotoxicity following the cerebral ischemic insult [31]. Furthermore, ethanol may also be neuroprotective through vasodilation and a subsequent increase in reperfusion, as it has been shown to cause vasodilation in low doses [32]. Wang *et al.* [33] used the MCAO model in adult rats to show a concentration dependent neuroprotection of ethanol. In this study they found that pre-administration (i.e., before surgery) was the most neuroprotective time of administration for ethanol and that 1mg/kg was the minimum efficacious dose. They also found that the therapeutic window for a 2mg/kg dose of ethanol was large, with administrations at 0h, 2h and 3h after the injury being equally efficacious; the 4h administration did reduce the infarction volume but less so than the 2h or 3h time-points.

ACTIVE VEHICLES AS CONFOUNDS

The standard experimental method for testing the efficacy of potential neuroprotective agents is to compare a drug treatment group with a vehicle group, and analyze the results with a null hypothesis test. Although this is an appropriate experimental approach to the problem, the reliability of null hypothesis testing in neuroscience is often limited by a lack of statistical power [34]. Statistical power is not only a function of sample size and within-group variation, but also of the effect size (the standardized mean difference in outcome between the treatment and control group) [35]. Because of this, if the vehicles used in the control group have neuroprotective properties, then this will reduce the ability of the experiment to detect any positive effect by the drug under investigation. Hence, the rate of Type II error will increase, and potentially useful drugs may be discarded.

It could be argued that a powerful neuroprotective agent should have additive effects with any active vehicle. However, the relationship is just as likely to be competitive, given that the amount of protection that can be provided by treatment is finite, and that it is possible that the vehicle and drug could overlap with respect to their mechanisms of

action. This would reduce the power of the experiment even further. Given that reduction in brain damage is likely to be subtle rather than dramatic for any true neuroprotectants, this is a serious problem for drug testing. One possible solution may be to use a factorial experimental design with multiple vehicles, such that the drug is tested with two different vehicles against the two vehicles alone. A study of the interactions between the vehicles and the drug with respect to reduction in brain damage could tease apart the relative contributions of the drug and the vehicles.

Another possible solution is to combine several vehicle compounds – each at a low concentration – into a mix, such that each is likely to be below the threshold concentration for neuroprotective activity. Combinations of solvents are sometimes used for drug vehicles, for example DMSO, E-CL, and ethanol (henceforth referred to as "DCE") are commonly used to deliver cannabinoids. DCE contains three of the most common solvents used in vehicles for lipophilic drug research [7, 36-38]. Each individual solvent is used at a concentration that is expected be below a threshold concentration for its particular biological activity. For example, to dissolve drugs as lipophilic as cannabinoids at least a 15% ethanol solution isusually required. This corresponds to a high dose of ethanol, and can be dramatically reduced by combining the ethanol with DMSO and E-CL. However, the use of multiple solvents is not without problems as by using several solvents the *number* of possible physiological side effects may increase, even as the severity of each side effect is reduced.

ACTIVE VEHICLES AS OPPORTUNITIES

Although active drug vehicles can be an experimental nuisance and confound the testing of drugs of interest, they can also be interesting as potential leads in their own right. Although such a compound as DMSO has a long history of investigation, and has been found to have pleiotropic effects – some potentially harmful [39] – the same cannot be said for other drug vehicles. Cyclodextrins are under investigation as potential medicines; for example as a treatment for lysosomal storage disease. However, the details of the actions of cyclodextrins on the nervous system are poorly understood and could reward further investigation. E-CL would seem to be a particularly good candidate for further study; as it is a heterogeneous complex of lipids and other molecules, chemical deconvolution and screening may reveal novel active compounds. The point is a general one – wherever drug vehicles cause a problem in testing a drug, there may also be an opportunity for incidental discovery.

CONCLUSION

Of the four solvents and carriers commonly used in preclinical research for lipophilic drugs that have been reviewed in this article, there is evidence for potentially mild to moderate neuroprotective properties for all of them. Whether this conclusion will withstand further scrutiny remains to be determined, but as of the time of writing the evidence suggests that any experiment which employs one or more of these compounds as drug vehicles may be confounded and thus underpowered. The main methodological outcome of this should be an increase in Type II error rates, where potentially useful drugs fail to show an effect that is sufficiently greater than the vehicle effect to reach statistical significance. This represents lost opportunities, and so careful consideration should be given in the design of experiments to reduce these problems. However, the seeming neuroprotective effects of some of these compounds remains poorly studied and understood, and could represent research opportunities in themselves.

CONFLICT OF INTERESTS

The authors confirm that this article content has no conflicts of interest.

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