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Research report

Long term cerebroprotective effects of dexanabinol in a model of focal cerebral ischemia

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Abstract

In order to test the long-term cerebroprotective effects of dexanabinol, a synthetic non-competitive NMDA antagonist that also has anti-TNFα effects, spontaneously hypertensive rats underwent permanent middle cerebral artery occlusion (PMCAO). Rats were given vehicle or dexanabinol (4.5 mg/kg) 1, 3 or 6 h after PMCAO. The research consisted of 2 stages. In the short-term set of experiments animals ($n=5$/group), were tested with a motor disability scale 24 h post PMCAO, then sacrificed and the infarct volume was measured using 2,3,5-Triphenyltetrazolium chloride (TTC) staining. In the long-term set of experiments the rats ($n=7$/group) were examined daily with a motor disability scale up to 30 days after PMCAO and then sacrificed and infarct volumes were determined using TTC staining. Motor scores were significantly improved in the dexanabinol treated rats ($P<0.05$ for all groups) at all the time points examined. Infarct volumes were significantly reduced 24 h after PMCAO in the groups treated 1 or 3 h, but not 6 h after PMCAO compared with vehicle (Mean±S.D., 11.5±2.02, 12±3.2 and 14.4±2.4% vs. 20.8±1.3% hemispheric volume respectively). The lesions remained significantly smaller in the dexanabinol groups 30 days after PMCAO (Mean±S.D., 24.49±1.9% vs. 8.1±0.6, 11.1±2.3 and 13.8±2.5% hemispheric volume in animals treated with vehicle vs. dexanabinol 1, 3 or 6 h after PMCAO respectively; $P<0.05$ for all). In conclusion, the extended therapeutic window and the multi-mechanistic durable neuroprotective effects of dexanabinol make it a promising candidate for future stroke therapy.

Theme: Disorders of the nervous system

Topic: Cerebral ischemia

Keywords: Cerebral ischemia; Dexanabinol; HU-211; NMDA; Cytokines; Free radical

1. Introduction

Cerebral ischemia may lead to irreversible neuronal damage at the core of the ischemic focus but neuronal dysfunction may be reversible in the surrounding area, the penumbra. Ischemic damage is multifactorial and involves excitotoxicity [1], reactive oxygen species (ROS, [20]), inflammation [2,12] and apoptosis [11,18].

The synthetic cannabinoid (+)-(3*S*,4*S*)-7-hydroxy-Δ^6-tetrahydrocannabinol-1,1-dimethylheptyl (dexanabinol; previously called HU-211) is a promising neuroprotective compound, which was previously demonstrated to reduce infarct volume significantly when given to rats after induction of cerebral ischemia [17]. It is a non-competitive antagonist of the NMDA glutamate receptor with neuroprotective capabilities in both trauma and ischemic conditions [6,13,15,22] that also protects blood–brain barrier integrity [23]. Dexanabinol has been recently shown to abrogate the effects of TNFα and IL-1 in a rat head trauma model and in an endotoxic shock model [21] as well as in cerebral ischemia [17]. These qualities may have salutary effects against reperfusion injury and exitotoxicity as well as against inflammation and free radical-induced damage. Unlike other NMDA antagonists, dexanabinol is safe and devoid of psychotropic side effects [8]. The relevance of dexanabinol for clinical use was demonstrated in a study

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on closed head injury in rats, in which a therapeutic window of 4–6 h was reported and its long-term protective effect was demonstrated [22]. However, previous reports on its protection after ischemia documented only the short-term protective effects [3,7,9]. Therefore, we now report on the long term cerebroprotective effects of dexanabinol in a rat model of permanent middle cerebral artery occlusion (PMCAO), as reflected by reducing lesion size as well as by facilitating recovery of motor and cognitive functions.

2. Materials and methods

2.1. Animals

Male spontaneous hypertensive rats (SHR) 13 weeks of age were obtained from the Tel Aviv University animal facility. All animals were housed in the animal care facility of the Hebrew University Faculty of Medicine in compliance with the standard guidelines for animal care. The animal care committee of the Hebrew University Faculty of Medicine approved all experiments. Animals were given free access to food and water to the night before surgery and at all times following recovery from anesthesia.

2.2. Surgical procedure and drug administration

Permanent middle cerebral artery occlusion (PMCAO) is a highly reproducible method for induction of cerebral ischemia that has been previously described [4,5]. Following proper anesthesia with intraperitoneal Phenobarbital (30 mg/kg), the animals were allowed to breathe spontaneously and were not ventilated. The left femoral artery was cannulated for invasive measurement of blood pressure (Biopac MP 100-TSD104A, Biopac Systems, Santa Barbara, CA). Temperature, heart rate and oxygen saturation were measured throughout the experiment and for 4 h after drug administration using external sensors (Biopac Oxy 100 and SKT 100, Biopac Systems, Santa Barbara, CA). Rats were placed in a tiltable stereotaxic head holder under a surgical microscope. The temporozygomatic suture was exposed after dissection of the temporalis muscle. A 0.5 mm burr hole was drilled with a dental foot-operated hand held drill. The middle cerebral artery was then exposed all the way down to its junction with the inferior cerebral vein and the dura was reflected off the brain. Finally the artery was occluded and cut by electrocoagulation as it was carefully traced off the brain surface. Body temperature was measured throughout surgery with a rectal probe and was maintained at 37 ± 0.5°C by using a heating lamp. Brain temperature was not monitored in this experiment since previous data suggests that this drug does not affect brain temperature [8]. Blood pressure, heart rate and oxygen saturation were also monitored throughout the surgical procedure and until awakening. The animals were injected with dexanabinol (4.5 mg/kg) or vehicle i.v. via the tail vein by one of the investigators 1, 3 or 6 h after onset of ischemia ($n=12$/group). This dose was specifically chosen, since it was previously shown in dose response preliminary experiments to provide the largest amount of cerebral protection without leading to any side effects [15,17,23]. Sham operated animals ($n=4$) that underwent craniotomy and exposure of the artery without occlusion were used as control for possible surgical damage.

2.3. Motor disability evaluation

All evaluations were performed by one of the investigators (G.L.) that was blinded to the treatment regimen. Rats were examined daily before sacrifice with a motor disability scale according to the method described by Bederson et. al. [5] with slight modifications. Animals were scored 1 point for each of the following parameters: flexion of the forelimb contralateral to the stroke when hung by the tail, extension of the contralateral hindlimb when pulled from table, and rotation to the paretic side against resistance. Additionally, 1 point was scored for circling motion to the paretic side when attempting to walk, 2 points for failure to walk out of a circle 30 cm in diameter within 20 s, and 3 points for total inability to walk. Thus, an animal with a maximal deficit scored 6 points and an animal with no deficit scored 0 points. Clinical evaluations were performed on day 1 after surgery in the short-term group and on days 1–5, 10, 20 and 25–30 after PMCAO in the long-term group. The animals were also weighed daily up to their sacrifice.

2.4. Infarct size

All evaluations were performed by one of the investigators (R.R.L.) that was blinded to the treatment regimen. In the short-term set of experiments the animals ($n=5$/group) were anesthetized and sacrificed with excess phenobarbital injected into the heart 24 h after the surgery. The brain was carefully removed and sliced to 2 mm slices using a mold. The slices were stained with 2,3,5-Triphenyltetrazolium chloride (TTC, 2% solution in PBS) for 8 h and preserved in 3.7% formaldehyde. They were photographed on-line with an image acquirement (Lis-700, APPLItec, Rehovot, Israel) system. Image analysis software (Sigma Scan Pro, SPSS inc. Richmond CA) was used for the estimation of the infarcted area. We used the formulation devised by Lin et.al. [19] to determine the infarct volume, in order to control for the effects of edema (corrected infarct volume = left hemisphere − [right hemisphere − right infarct]/left hemisphere). The results are expressed as a percentage of the contralateral hemisphere.

In the long-term set of experiments rats ($n=7$/group)

were sacrificed 30 days after PMCAO and the infarct size determined as the difference between the volumes of the 2 hemispheres divided by the volume of the normal hemisphere (infarct volume = (infarcted hemisphere − normal hemisphere)/normal hemisphere). This formulation was used since at this time point the infarcted zone has undergone necrosis and liquefied and therefore could not be measured directly. Therefore, measurements may slightly overestimate the actual infarct volume due to the effect of shrinkage of the ipsilateral hemisphere that occurs after PMCAO. However, given the similarity in size between the 2 hemispheres in a normal animal, the difference between the volumes of the 2 hemispheres at 30 days was thought to closely resemble the volume of the absent infarcted tissue.

2.5. Statistical analysis

Analysis was performed with the Sigma-Stat software package (SPSS, Richmond, CA). Data are presented as mean±S.D. or as mean±S.E. as indicated in the legends. Differences between the groups were compared using the one-way analysis of variance (ANOVA) with Dunnett's correction. Day-to-day differences in motor scores and water maze latency times within each group were compared with ANOVA on repeated measures.

3. Results

3.1. Physiologic variables (Table 1)

Blood pressure, heart rate, oxygen saturation and temperature remained constant in all animals throughout the surgery, drug administration and up to 3 h thereafter.

3.2. Weight (Table 1)

There were no statistically significant differences in weight measurements between the study groups at the individual time points examined. Mean weight loss from baseline to the 10th day after PMCAO was larger in the vehicle group. A statistically significant loss of weight ($P<0.05$ one-way ANOVA on repeated measures) was demonstrated from day 1 to 10 in the vehicle group, but not in the dexanabinol groups.

3.3. Motor disability scores (Fig. 1)

Sham operated rats had no motor impairment. In the short-term set of experiments motor scores were significantly lower (i.e. the animals were less disabled) in all the active treatment groups as compared with vehicle ($P<0.05$ for all groups). Motor scores were lowest in the group that received dexanabinol 1 h after PMCAO as compared with

Table 1
Changes in weight and physiological variables of dexanabinol and vehicle treated animals following cerebral ischemia

	Dexa 1h	Dexa 3h	Dexa 6h	Vehicle	*P*
Weight (g):					
Days post surgery					
0	310±25.4	314±34	335.2±24.3	307.6±10	>0.05
1	276.4±10.7	274±36.4	288.6±10.7	286±8.2	>0.05
5	263.6±32.1	272.7±14	303.2±19	254±15.5	>0.05
10	260.7±27.3	283.7±10	304±22	270±19.7	>0.05
20	291.7±36.3	308±52.6	330±20	283.1±12	>0.05
30	291.7±14.8	304±58.1	344±19	286±17.5	>0.05
BP (MAP±S.D.):					
Baseline	127.3±11	126.1±12.1	128.5±11	131.7±8.9	>0.05
30 min	121.5±9.3	119.5±13.6	122.3±1.9	128.2±9.1	>0.05
60 min	131.5±10.6	125.5±7.7	133.2±8.4	134.5±7.3	>0.05
HR (±S.D.):					
Baseline	72.6±4	69.2±3.3	78.3±1.4	70.3±4.4	>0.05
30 min	67.2±3.4	66.3±4.1	68.5±2.4	76.8±6.2	>0.05
60 min	69.2±2.2	59.9±11.3	68.6±3	69.3±3.5	>0.05
O_2 SAT (%±S.D.):					
Baseline	98.9±1.9	98.2±1.8	97.2±2.1	98.3±1.3	>0.05
30 min	98.2±1.5	97.3±2.5	98.3±3.6	98.4±1.5	>0.05
60 min	97.2±2.4	98±1.2	98.2±1.2	97.3±1.9	>0.05

Data are presented as mean±S.D.
Dexa 1 h, Dexa 3 h, Dexa 6 h – dexanabinol administered 1, 3 or 6 h, respectively, after PMCAO.
Data for physiological variables is presented for baseline, 30 and 60 min following ischemic onset.
BP – blood pressure, HR – heart rate, SAT – saturation.
P = *P* value as measured one-way ANOVA with Dunnett's correction.

those that received the drug 3 and 6 h after PMCAO when tested on the first post-operative day. At later time points motor disability scores were significantly lower and overlapping in all the dexanabinol-treated animals when compared with those of the vehicle treated animals. Overall the mean reduction in motor disability measured 24 h post stroke was 22, 44 and 61% of that experienced by animals treated with vehicle in the animals that received the drug at 1, 3 and 6 h following PMCAO respectively (mean score±S.D., 0.8±1.03, 1.6±0.6 and 2.2±0.37 vs. 3.6±1.2 respectively). In the long-term experiment animals treated with dexanabinol had better scores on all examination days as compared with those that were treated with vehicle (Fig. 1). In the long-term group the reduction in motor disability was to ~40% of that experienced by the vehicle group on the 30th day after the stroke for animals treated with dexanabinol 1, 3 or 6 h following PMCAO (mean score±S.D., 1.3±0.6, 1.4±0.2 and 1.2±0.2 vs. 3.29±0.5).

3.4. Infarct volumes (Fig. 2)

Sham operated rats had no histological evidence for infarction. The observed infarcts in the dexanabinol and vehicle animals involved cortical areas only and spared the subcortical structures. At 24 h after PMCAO rats that received dexanabinol at 1 or 3 h after PMCAO showed a significant reduction in infarct volumes as compared with vehicle treated animals (mean±S.D., 11.5±2.02% and 12±3.2% vs. 20.8±1.3% hemispheric volume, $P<0.05$). Although rats treated 6 h after PMCAO had smaller infarcts as compared with vehicle (mean±S.D., 14.4±2.4%) this difference did not reach statistical significance. The absolute reductions in infarct volumes compared with vehicle were 45, 42 and 31% in animals treated at the 1, 3 and 6 h markers after PMCAO in the short term set of experiments. At 30 days post PMCAO the infarct size was significantly smaller in dexanabinol treated animals at 1,3 or 6 h after the stroke as compared with vehicle-injected rats (8.1±0.6, 11.13±2.3 and 13.8±2.49 vs. 24.49±1.9% hemispheric volume respectively, $P<0.05$ for all). Compared with vehicle treated rats the infarct volume reductions were of 67, 55 and 44% in animals treated within 1, 3 and 6 h of PMCAO respectively in the long-term experiments.

While total lesion volumes increased from day 1 post PMCAO to day 30 post PMCAO for vehicle injected rats, they were slightly reduced in dexanabinol treated rats. However, changes in lesion volumes between days 1 and 30 after PMCAO were not significantly different within the dexanabinol or vehicle groups.

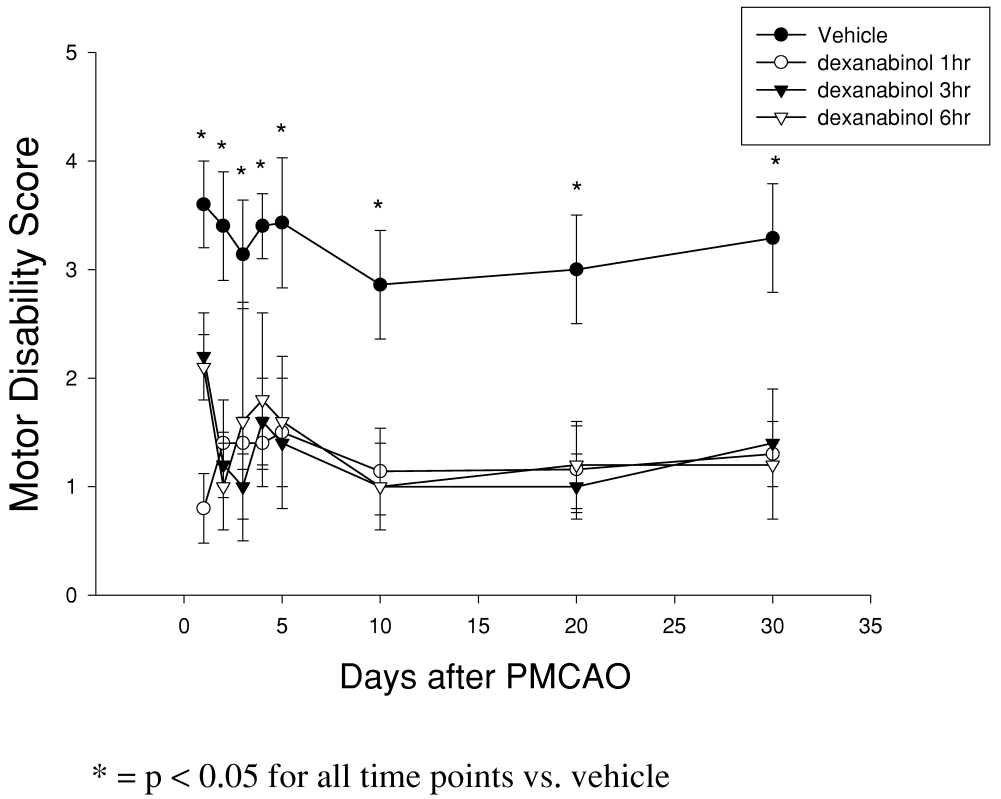

Fig. 1. Motor scores in dexanabinol and vehicle treated rats after PMCAO. Rats were examined with a motor scale at the time points indicated in the figure starting at 24 h after PMCAO. Rats received 0.3 ml Saline (vehicle) or dexanabinol (4.5 mg/kg) I.V. 1, 3 or 6 h after PMCAO. Data is presented as mean±S.E.M. ($n=5$ per time point). $P<0.05$ (ANOVA) for all time points tested. PMCAO – permanent middle cerebral artery occlusion.

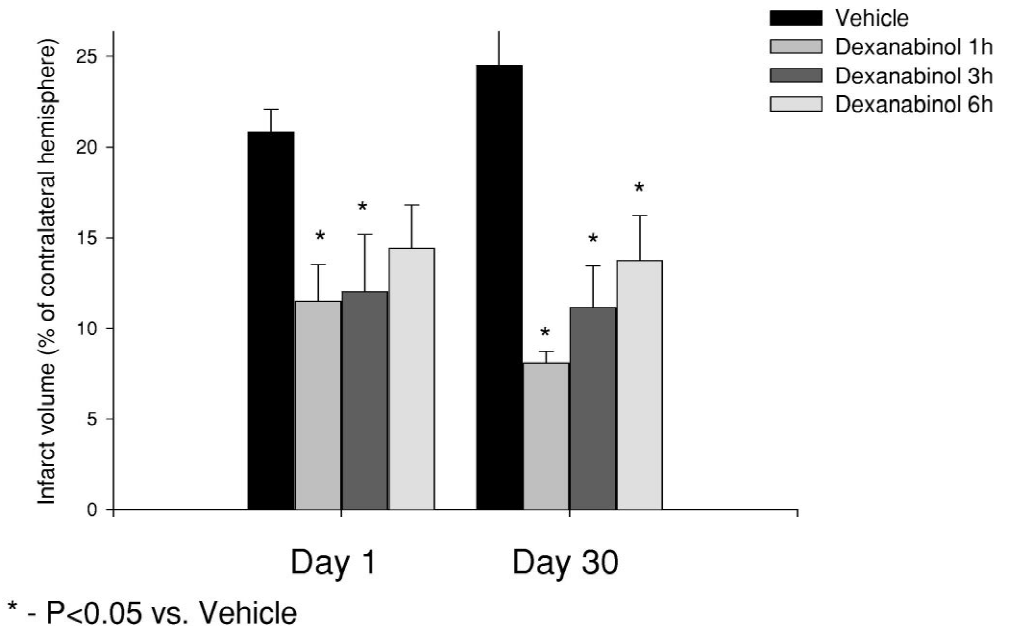

Fig. 2. Infarct volumes in dexanabinol and vehicle-treated rats. Animals were sacrificed 1 or 30 days after PMCAO ($n=5$ or 7 per group, per time point respectively). The brains were sliced and stained with TTC and the infarcted volumes were measured with an image analysis system. Data is presented as mean±S.E.M. Day 1 results – $P<0.05$ (ANOVA) for the animals treated 1 or 3 h after PMCAO and $P>0.05$ (ANOVA) for animals treated 6 h after PMCAO compared with vehicle treated rats. Day 30 results – $P<0.05$ (ANOVA) for dexanabinol treated rats at all time points compared with vehicle. PMCAO – permanent middle cerebral artery occlusion.

4. Discussion

Cell death in the ischemic penumbra involves several pathologic processes including excitotoxity, oxidative stress, inflammation and apoptosis [16]. Cerebroprotective agents active against only one of these mechanisms have generally failed to show positive results in clinical trials probably because the other unblocked mechanisms bring about cell death. Therefore, drugs interfering with more than one of these damaging mechanisms might have significant advantages in neuronal protection. Dexanabinol is an active non-competitive NMDA antagonist [14,15] that has been shown to possess anti-TNFα [17,21] and anti-NO [14] effects in models of endotoxic shock, ischemia, and head trauma [17,22,23]. Because all of the above mechanisms of cell death are induced shortly after the onset of ischemia it may not be possible to dissect out along the time axis, the inhibition of which of these toxic mediators constitutes the bulk of the anti-ischemic properties of dexanabinol. However, because the dose used in the current study is well within the range that was shown to inhibit separately each of these mediators it is conceivable that the anti-ischemic effects of dexanabinol are multi-faceted and include all of the above mechanisms.

In the present study we were able to show that dexanabinol improves recovery of motor functions and significantly reduces infarct volumes in both the short and long terms following focal cerebral ischemia. It should be noted that the sustained protection, evident even at 30 days after the insult was obtained after a single administration of the drug, at 1, 3 or 6 h after induction of PMCAO. This agrees with earlier reports on long-term (30 days) effect of dexanabinol in a rat model of closed head injury, where single administration even at 4 h post injury was as effective as repeated dosages [22].

Our results in the short-term experiments showed that dexanabinol reduced infarct volumes by 42–45% when administered 1 or 3 h after PMCAO. This reduction is of the same order of that observed in our previous report of the acute cerebroprotective effects of dexanabinol in this model and therefore, corroborates our early results [17]. Lesion volumes were also reduced when the drug was administered 6 h after the surgery but this reduction just failed to reach statistical significance. Thus, the drug appears to be active when given up to 3 h after the onset of ischemia and still shows a moderate degree of activity even when given at later time points after PMCAO. Our long-term results show that the reductions in infarct volumes observed 24 h post PMCAO remains significant with time. The best results were obtained when the drug was given 1-h post PMCAO. Infarct volumes decreased to a lesser degree in animals treated at 3 h and to an even lesser, but still significant, degree in animals treated within 6 h. These results re-emphasize the importance of early treatment after the onset of ischemia. Our results delineate a therapeutic window of 3–6 h after the onset of ischemia

for dexanabinol. This is of particular clinical relevance since most stroke patients do not seek very early medical assistance but rather delay their arrival to the hospital for several hours.

Interestingly, we observed a slight increase in infarct volume from day 1 to 30 after PMCAO in our study in the vehicle group whereas commonly a slight decrease in volumes was to be expected. This finding can be explained by the fact that the method we used for estimation of infarct size at 30 days post PMCAO relies on the macroscopic similarity in the size of the two hemispheres as outlined in the method section. However, since it does not measure directly the infarcted tissue volume, as this tissue has liquefied by that time point, it does not correct for the presence of atrophy of the ipsilateral hemisphere. Therefore, it may slightly over-estimate the infarct size. Nevertheless, the changes in lesion size between days 1 and 30 after PMCAO were not statistically significant.

The reduction in infarct volumes was accompanied by a lesser degree of motor disability in the long-term. Importantly, the actual reduction in infarct size was smaller than the degree of improvement in the motor score observed at 24 h after the stroke. This implies that the actual lesion size may not correlate directly with the clinical deficits experienced by the animals. Rather, the degree of motor deficits is probably related to specific reduction of the lesion size at the motor and pre-motor areas. Indeed, the experimental model used in this study uses occlusion of the distal portion of the middle cerebral artery, which provides the blood supply to these motor areas.

In the current set of experiments we used the surgical PMCAO model. This model involves permanent occlusion of the artery and therefore, drug delivery to the penumbral zone is limited to the collateral circulation and to transvenous access. These limitations guarantee that only compounds with robust anti-ischemic properties will be truly cerebroprotective in this model. Thus, it is possible that in other models using transient ischemia, dexanabinol could have an even larger effect [7].

Importantly, dexanabinol was shown to have a potent cerebroprotective effect against traumatic brain damage in phase 2 clinical trials in humans (Knoller et al., data presented at the American Congress of Neurological Surgeons, October 7, 1998, Seattle, Washington) and a phase 3 clinical trial is ongoing. A growing body of evidence suggests that many similarities exist regarding neuronal death mechanisms between traumatic and ischemic damage. Therefore, the fact that dexanabinol is effective in reducing traumatic brain damage in humans suggests that it may also have important protective effects against ischemic stroke.

In this set of experiments we used a dose of dexanabinol that was shown to be protective without causing any significant side effects. Specifically, at the dose of dexanbinol selected for this study no changes were observed in heart rate, blood pressure or any other physiologic variables [22]. Furthermore, this compound was studied in various neurotoxic paradigms both in vitro (at a dose range of 1–50 μM) and in vivo (at a dose range of 1.25–14 mg/kg, depending upon species and route of administration) [9]. Dexanabinol does not act as an agonist of the endogenous cannabinoid receptor Cb1 (in contrast with the actions of the racemate HU-210), and binds to a specific site at the NMDA receptor [13]. Therefore, it is devoid of significant psychotropic side effects [8]. Furthermore, in contrast to other NMDA antagonists such as MK-801and CB1 agonists such as HU-210 it also does not change the brain's temperature excluding hypothermia as a possible potential neuroprotective mechanism [8]. The safety profile of dexanabinol in phase 1 [10] and 2 clinical trials in humans is also very favorable with only minor and insignificant side effects observed (Knoller et al., data presented at the American Congress of Neurological Surgeons, October 7, 1998, Seattle, Washington). Safety issues are extremely important when considering the use of NMDA antagonists and therefore, the preliminary human clinical data obtained with dexanabinol is very reassuring.

In conclusion, we found that dexanabinol exerts potent long-term cerebral protection against ischemic damage with an extended therapeutic window. Its use provides beneficial effects on motor measures and it significantly reduces infarct volumes. We believe that taking these points together with dexanabinol's high safety profile outlines this drug as an important adjunct to current anti-ischemic therapies. Thus, the combination of tissue reperfusion with thrombolysis and a potent, pluripotent cerebroprotective agent such as dexanabinol warrants further research as a new stroke therapy.

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