Efficacy comparison of the novel water-soluble propofol prodrug HX0969w and fospropofol in mice and rats

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Editor’s key points

- Fospropofol is approved by the Federal Drug Administration but its metabolite, formaldehyde, is not considered to be ideal.
- In this study, a similar water-soluble compound HX0979w was synthesized, which had gamma-hydroxybutyrate as a metabolite.
- HX0969w released propofol and was as effective as fospropofol in rats and mice.
- Further studies are needed, but HX0969w may be a safer alternative to fospropofol.

Background. HX0969w is a novel water-soluble prodrug designed to release propofol and gamma-hydroxybutyrate (GHB) and has a sedative–hypnotic effect. This study was performed to compare the efficacy of HX0969w with fospropofol in mice and rats.

Methods. We performed hydrolysis studies in the plasma from mice and rats. The half-maximal effective doses (ED50) and half-maximal lethal doses (LD50) of fospropofol and HX0969w were determined. A pharmacodynamics comparison of these two compounds was also performed. Time to loss of righting reflex, time to return of righting reflex, recovery time, and adverse effects were recorded.

Results. The hydrolysis studies demonstrated that HX0969w released propofol as expected. HX0969w ED50 values in mice and rats were 133.03 and 53.79 mg kg⁻¹, respectively, and LD50 values were 607.11 and 283.79 mg kg⁻¹, respectively. The calculated therapeutic index (TI), safety index (SI), and certain safety factor (CSF) of HX0969w were 4.56, 3.33, and 2.92 for mice, and 5.28, 3.94, and 3.49 for rats, respectively. The pharmacodynamic comparison studies suggest that HX0969w has a longer onset time and shorter duration than fospropofol.

Conclusions. Similar to fospropofol, HX0969w is an effective, water-soluble prodrug that is capable of inducing a sedative–hypnotic effect in mice and rats. Unlike fospropofol, HX0969w releases GHB instead of formaldehyde. Further studies regarding the efficacy and safety of HX0969w are necessary.

Keywords: anaesthetics i.v., propofol; gamma-hydroxybutyrate; potency, anaesthetic, ED50; water-soluble prodrug

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Propofol, an i.v. sedative–hypnotic agent with rapid onset and recovery, is widely used to induce and maintain general anaesthesia and long-term sedation in intensive care units (ICUs). However, its lipid-based formulation has disadvantages, including emulsion instability, hyperlipidaemia, and injection pain. New propofol formulations that do not have these disadvantages are currently being developed (e.g. aqueous formulations of propofol prodrugs).

Propofol phosphate is a water-soluble propofol prodrug that can be enzymatically converted to propofol and inorganic phosphate, but its long onset and duration make it unsuitable for anaesthesia. Fospropofol disodium (Luseda®) is the only water-soluble propofol prodrug approved by the Federal Drug Administration (FDA) in the USA. Though its metabolite, formaldehyde, is an endogenous compound which has been confirmed not to be accumulated in the body after administration of fospropofol, it is considered better for the propofol prodrug not to have formaldehyde as a metabolite. Therefore, novel propofol prodrugs that do not incorporate formaldehyde have been synthesized. Ethyl dioxy phosphate is a water-soluble propofol prodrug with a metabolite of acetaldehyde, which is considered not as detrimental as formaldehyde.

Unlike formaldehyde and acetaldehyde, gamma-hydroxybutyrate (GHB) is an endogenous neurotransmitter that is rapidly and completely converted into CO₂ and H₂O through the Krebs cycle. Thus, GHB is considered a more suitable metabolite of a propofol prodrug than formaldehyde or acetaldehyde. We synthesized HX0969w, a novel water-soluble prodrug composed of propropofol, GHB and a phosphate group, with intramolecular cyclization reactions. On the basis of these reactions, we hypothesized that HX0969w would release propofol and a GHB by-product (Fig. 1) and induce a sedative–hypnotic effect similar to that of fospropofol.

In this study, we assessed the efficacy of HX0969w in mice and rats. Hydrolysis studies in the plasma were performed to ascertain whether HX0969w can release propofol as hypothesized and to compare it to fospropofol. The median effective
dose (ED$_{50}$) and median lethal dose (LD$_{50}$) of both compounds were evaluated and compared. Likewise, we compared the pharmacodynamic properties of equivalent doses of HX0969w and fospropofol in mice and rats.

**Methods**

**Chemicals and reagents**

HX0969w (molecular weight, 389.11 g mol$^{-1}$) and fospropofol (molecular weight, 332.28 g mol$^{-1}$) were synthesized according to the protocols described in their patents.$^{19}$

Solutions of HX0969w (10, 20, and 80 mg ml$^{-1}$) and fospropofol (10, 15, and 50 mg ml$^{-1}$) were prepared using 0.9% normal saline.

**In vitro studies**

**Hydrolysis study in rodent plasma**

The objective of the hydrolysis study was to investigate the release of propofol from HX0969w and fospropofol, respectively. Stability of HX0969w and fospropofol in normal saline was determined at 37°C for 5 h before study. HX0969w (10 mg ml$^{-1}$, 0.97 ml) or fospropofol (10 mg ml$^{-1}$, 0.83 ml) was added to pre-heated mouse or rat plasma, respectively, and the mixture (5 ml, 5 μmol ml$^{-1}$) was shaken in a 37°C water bath for 3 h.$^{20}$ The samples (100 μl) withdrawn from the mixture were added to 900 μl of methanol in order to deproteinize the plasma at 0, 1, 3, 5, 7, 10, 20, 30, 60, 120, and 180 min. Then, the mixtures underwent vortex for 5 s and centrifugation for 13 min at 12 000 × g. The supernatants (10 μl) were, respectively, collected for propofol analysis with high-performance liquid chromatography (HPLC) (Agilent 1100 series, Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of methanol–water (70:30), on an Eclipse Plus C$_{18}$ reversed-phase column kept at 25°C (150 × 4.6 mm, particle size of 5 μm, Agilent Technologies) with a guard column (Phenomenex, Torrance, CA, USA). The flow rate was 1 ml min$^{-1}$, and the UV absorbance detector was set at 272 nm. Both the limit of quantification and detection for propofol were 0.2 μg ml$^{-1}$. The retention time for propofol was ~7 min. The standard curve ranged from 0.2 to 100 μg ml$^{-1}$, with $R^2 ≥ 0.99$. The inter- and intra-precision at three concentrations (0.4, 8, and 80 μg ml$^{-1}$) were in the range...

![Chemical structure and the possible enzymatic hydrolysis path of HX0969w.](attachment:chemical_structure.png)
between 0.55 and 7.53% coefficient of variation (Supplementary Tables S2 and S3). The intra- and inter-batch accuracy for propofol at the three concentrations was 95.55–107.03 and 98.64–102.33%, respectively (Supplementary Tables S2 and S3). The absolute recovery was in the range of 83.53–96.87%. The selectivity of the HPLC method was shown in Supplementary Figure S1.

In vivo studies

The animal studies were approved by the Committee of Scientific Research and the Committee of Animal Care of the West China Hospital, Sichuan University, China. Adult male Kunming mice and Sprague–Dawley rats were purchased from Chengdu Dassy Biological Technology Co. Ltd (Chengdu, China) and cared for in accordance with the Guide for Care and Use of Laboratory Animals published by US National Institute of Health (NIH Publications No. 80-23, revised 1996). Animals were housed 5 per cage under standard conditions: with food and water ad libitum. Animals were allowed to acclimatize for a period of 1 week. Mice (18–24 g) and rats (180–240 g) were used.

The in vivo studies included the determination of the median effective doses (ED50) and lethal doses (LD50) of HX0969w and fospropofol, and also a pharmacodynamic comparison of the two compounds. Before injection, the animals were randomized using a random number protocol with Excel 2003 (Microsoft, USA) and divided into groups (n=10). The allocated dose was randomized. Before injection, the animal was weighed and placed into a restrainer. Drugs were given i.v. into the tail vein. The injection took ~10 s for the experiments to determine ED50 and pharmacodynamics, and 10–30 s for LD50 measurement. Animals were not used for multiple experiments and were killed with an overdose of pentobarbital i.p. at the end of the study.

Determination of ED50 and LD50

The prodrug doses used in this section were results of preliminary experiment. For mice, the doses of HX0969w for ED50 measurement were 106.25, 115.63, 125.00, 136.03, 147.06, or 173.01 mg kg\(^{-1}\); doses of fospropofol were 60.59, 71.28, 77.57, 83.86, 98.66, or 116.07 mg kg\(^{-1}\). For LD50 determination, high doses of HX0969w (486.74, 572.64, 597.90, 623.16, 673.69, or 792.58 mg kg\(^{-1}\)) and fospropofol (213.51, 251.19, 273.35, 295.52, 321.59, or 347.67 mg kg\(^{-1}\)) were administered.

To determine the ED50 of HX0969w and fospropofol in the rats, animals received doses of HX0969w (44.03, 47.92, 51.80, 54.08, 56.37, or 60.94 mg kg\(^{-1}\)) or fospropofol (34.00, 35.50, 37.00, 40.00, 43.53, 47.06, or 55.36 mg kg\(^{-1}\)). For the LD50 study, HX0969w was given in doses of 239.01, 260.10, 270.64, 281.19, 306.00, and 330.81 mg kg\(^{-1}\) and fospropofol in doses of 112.68, 132.57, 155.96, 169.72, 183.48, and 215.86 mg kg\(^{-1}\).

After injection, animals were removed from the restrainers and monitored by an observer blinded to dose or drug allocation. When animals exhibited ataxia, they were laid on their backs. Loss of righting reflex (LRR) for more than 30 s was considered induction of anaesthesia;21 –23 return of righting reflex (RRR) (righting themselves twice) was considered to be waking.24 Recovery was defined as regaining co-ordinated grooming or purposeful exploration.25 When the animals were observed to have stopped breathing, four electrodes were put on their forelimbs and hindlimbs to observe Electrocardiogram (ECG) waveforms. Disappearance of the ECG waveform was indicative of death during the LD50 measurement.26 The time to LRR, RRR, and recovery was recorded for each animal. Animals were allowed to breathe 80% oxygen spontaneously until awakening. Any significant adverse effects were recorded between drug administration and recovery or death. Body temperature was maintained at 36–38°C.

Comparison of HX0969w and fospropofol pharmacodynamic properties

Two groups of 10 animals (mice or rats) were used to compare the pharmacodynamic properties of HX0969w and fospropofol at doses two-fold ED50 values. Righting, corneal, pinna, and Hoffner reflexes were measured in all animals.21 The time to LRR, RRR, and recovery was recorded as described above. The
pinna reflex was measured by placing an i.v catheter in the animal’s ear canal, and the reflex was determined to be present if the animal shook its head. The corneal reflex was measured by touching the animal’s eye with a catheter, and the reflex was present if the animal blinked. The analgesic (Haffner) reflex was measured by pinching a hind foot with a forcep, and the reflex was considered to be present if the animal withdrew its foot. Body temperature was maintained at 36–38°C and any adverse effects were also recorded by a blinded observer.

**Data analysis**

The conversion percentages of HX0969w and fospropofol in the hydrolysis study were calculated by the following equation: conversion percentage = \( \frac{C_{\text{pro}}}{100/(178 \times 0.5)} \), where \( C_{\text{pro}} \) denoted the plasma concentrations of propofol assayed by HPLC. Estimates for ED\(_{50}\), LD\(_{50}\), ED\(_{95}\), ED\(_{99}\), LD\(_{1}\), and LD\(_{5}\) were calculated using a Probit analysis (SPSS 17.0 software package; SPSS, Chicago, IL, USA). Therapeutic index (TI), safety index (SI), and certain safety factor (CSF), which represented pharmacodynamic safety and safety margin, were calculated as LD\(_{50}/\text{ED}_{50}\), LD\(_{1}/\text{ED}_{95}\) and LD\(_{1}/\text{ED}_{99}\), respectively. For the pharmacodynamic comparison, time to LRR, duration of LRR (calculated as: time to return of LRR − time to LRR), and recovery time (time to recovery − time to return of LRR) for both drugs were analysed using Student’s t-test. Data are presented as the mean (SD). A \( P \)-value of <0.05 was considered statistically significant. The hydrolysis study curves and dose–response curves were generated with GraphPad Prism V5.0 software (San Diego, CA, USA).

**Results**

**In vitro studies**

Hydrolysis study in the mouse and rat plasma

Both HX0969w and fospropofol were stable in normal saline at 37°C for 5 h (Supplementary Table S1). At 3 h, the conversion percentages of HX0969w and fospropofol were 10.91 (0.07) and 12.62 (0.10)% in the mouse plasma, respectively (Fig. 2A). The conversion percentages of HX0969w and fospropofol were 39.71 (0.26) and 77.58 (0.72)% in the rat plasma, respectively (Fig. 2A). These results suggested that HX0969w was also capable of releasing propofol and that it may induce a sedative-hypnotic effect in mice and rats, but the propofol release of both HX0969w and fospropofol was slow. This was confirmed not to be affected by pH in a second hydrolysis study (Supplementary Table S4).

In vivo studies

Determination of ED\(_{50}\) and LD\(_{50}\)

The ED\(_{50}\) and LD\(_{50}\) of HX0969w were greater than those of fospropofol and reported values of propofol in both mice and rats (Table 1).22 26 In addition, the TI, SI, and CSF of HX0969w were greater than those of fospropofol and propofol. The dose–response curves were shown in Figures 3 and 4.

During the ED\(_{50}\) and LD\(_{50}\) measurements, the time to LRR for HX0969w was 1.12–3.95 min; duration of LRR ranged 4.12–127 min, and recovery time was 0–22.37 min. The time to LRR, duration of LRR, and recovery time of fospropofol were 0.9–3.68, 2.83–77, and 0–21.67 min, respectively.

After administration of HX0969w or fospropofol, animals showed decreased activity, ataxia, LRR, waking or death, and recovery. During the process, the main adverse reactions were limb and tail tremor in mice and nose secretion in rats. The animals were under deep anaesthesia with no response to pain stimulus (Haffner reflex) during the LD\(_{50}\) studies. All the animals that survived the LD\(_{50}\) measurement were observed for 15 days, and none showed evidence of acute toxicity.

**Pharmacodynamic comparison between HX0969w and fospropofol**

In mice, the time to LRR was significantly longer for HX0969w [1.55 (0.21) min] than that for fospropofol [1.17 (0.22) min, \( P < 0.05 \)], but the duration of LRR was significantly shorter for HX0969w [26.73 (7.89) min] compared with that of fospropofol [39.50 (9.58) min, \( P < 0.05 \)]. There was no difference in recovery time.

In rats, the time to LRR was longer for HX0969w [2.43 (0.35) min] than that for fospropofol [1.76 (0.23) min, \( P < 0.05 \)]. There was no difference in LRR duration or recovery time between HX0969w and fospropofol. The time to LRR, duration of LRR, and recovery time for each drug at the two-fold ED\(_{50}\) dose are given in Table 2.

Reflex responses (corneal, pinna, and Haffner) were present in all animals after injection. Tremor was noted in eight of the mice receiving fospropofol and five that were administered HX0969w. No tremor was observed in rats, though nose secretions were observed in seven of the fospropofol rats and two of the HX0969w rats. Cessation of breathing and cyanosis were seen in one rat when the LRR duration was tested, but was restored by chest massage, and may have been the result of a tracheal blockage because of nose secretions.

**Discussion**

HX0969w is a novel water-soluble propofol prodrug that will be developed as an alternative compound of propofol and fospropofol, which both have disadvantages. The hydroxybutyrate moiety on HX0969w initiates an intramolecular cyclization reaction after the hydrolysis of the phosphate group, releasing propofol. The results of the in vitro studies described in this article demonstrate that HX0969w can release propofol, which is consistent with our hypothesis. However, the in vitro data showed that the release of propofol from both HX0969w and fospropofol was too slow to be used in in vivo studies. The mismatch between the conditions of in vitro and in vivo release from this class of compounds (phosphate ester pro-drugs) was reported in a previous article.27 The reason might be that HX0969w was mainly metabolized by tissue alkaline phosphatase rather than plasma alkaline phosphatase. Thus, further studies are required to fully explore the metabolic characteristics of HX0969w.
The results of ED\textsubscript{50}, LD\textsubscript{50} and pharmacodynamic comparison studies have indicated that the required dose of HX0969w is greater and somewhat less potent than that of fospropofol, but the TI, SI, and CSF values of HX0969w are greater than those observed for fospropofol, indicating that HX0969w has both a wider safety margin and a therapeutic range than fospropofol. The TI values of HX0969w are comparable with the published values of propofol (4.5 for mice, 3.1 for...
The slow onset of HX0969w may be because of the speed of propofol and GHB release, both of which are mediated by the intramolecular cyclization reaction after enzymatic hydrolysis of the phosphate group (Fig. 1). This reaction may occur more slowly than the reaction for fospropofol that is initiated by methylal decomposition after the enzymatic hydrolysis of the phosphate group.

In addition to its use as a general anaesthetic, propofol is also widely used as a sedative in ICU patients’ bronchoscopic or endoscopic procedures. In spite of its advantages of the rapid onset, short duration, rapid recovery, and amnesic effect, propofol also has disadvantages, such as risks of hyperlipidaemia and bacterial contamination. Fospropofol, a water-soluble prodrug, was designed to eliminate these problems. Approved for monitored anaesthesia in adults undergoing diagnostic or therapeutic procedures, fospropofol has a delayed onset and a prolonged duration of action, which may be useful in clinical conditions where rapid onset of action is not necessary, but a more prolonged effect is desired. Fospropofol is completely metabolized by alkaline phosphatase to yield propofol, phosphate, and formaldehyde. HX0969w, however, releases propofol, phosphate,
and GHB instead of formaldehyde. As an endogenous compound that is found in many tissues, GHB may be advantageous because of its unique pharmacological activity. GHB is a Gamma-Aminobutyric Acid B and GHB receptor agonist with a short elimination half-life of ~20–60 min in rats (548 mg kg\(^{-1}\)) and human (12.5, 25, and 50 mg kg\(^{-1}\), orally). GHB can exert specific effects on the neuroendocrine system and sleep architecture.\(^{16-30-34}\) In addition, its potential anxiolytic and antidepressant properties may also be beneficial to ICU patients.\(^{15,28-35}\) GHB is also used to treat cataplexy in patients suffering from narcolepsy or alcohol withdrawal.\(^{15}\) Although not directly demonstrated in this study here, these properties may enrich the pharmacological properties and clinical applications of HX0969w. Previous studies have shown that GHB can be used for sedation and i.v. anaesthesia and has good respiratory or haemodynamic tolerance, compared with propofol.\(^{36-37}\) However, additive or synergistic effects of GHB combined with propofol have not been documented. In our study, additive or synergistic effects could not be confirmed, because two-fold ED\(_{50}\) of HX0969w (108 mg kg\(^{-1}\)) in rats was less than the sedative-hypnotic dose of GHB (>200 mg kg\(^{-1}\)) and greater than two-fold ED\(_{50}\) of propofol (11.78 mg kg\(^{-1}\)).\(^{26-33}\) In addition, this study used a single dose without determination of GHB and propofol concentration in vivo and further studies are required.

The main adverse effects observed in the study were tremor and nose secretions. Tremor may be a propofol-associated effect.\(^{38}\)

One limitation of this study was that GHB and prodrugs were not measured in the hydrolysis studies. The i.v. dose of GHB required to induce a sedative-hypnotic effect in rats was >200 mg kg\(^{-1}\), but the two-fold ED\(_{50}\) of HX0969w was 108 mg kg\(^{-1}\), indicating that the LRR in animals was mainly induced by propofol.\(^{33}\)

In summary, we successfully synthesized a novel, water-soluble propofol prodrug that releases propofol and GHB. Rodent studies suggest HX0969w has a wide safety margin and therapeutic range. However, further studies are required to fully characterize HX0969w before it can be considered for clinical use.

**Supplementary material**

Supplementary material is available at *British Journal of Anaesthesia* online.

**Authors’ contributions**

Experimental design was performed by Y.Z., J.L., and W.S.Z., in vitro studies finished by Y.Z. and Y.W., in vivo studies completed by Y.Z., J.Y., and Y.W., and the manuscript written by Y.Z. and W.S.Z.

**Declaration of interest**

J.Y., J.L., and W.S.Z. are original authors of the HX0969w patent (WO2011160268).

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