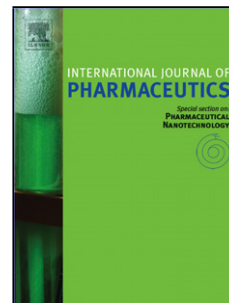# Accepted Manuscript

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In vivo pharmacological evaluation of a lactose-conjugated luteinizing hormone releasing hormone analogue

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Graphical abstract

Abstract:

In the current study, the efficacy and pharmacokinetic profile of lactose-conjugated luteinizing hormone releasing hormone (LHRH) was examined following oral administration in male rats. A rapid and sensitive liquid chromatography/mass spectrometry technique was developed and applied for measuring the concentration of lactose[Q^1][w^6]LHRH (compound **1**) in rat plasma in order to allow measurement of pharmacokinetic parameters. LH release was evaluated using a sandwich ELISA. Maximum serum concentration (C_{max}= 0.11 µg/ml) was reached at 2 h (T_{max}) following oral administration of the compound at 10 mg/kg. The half-life was determined to be 2.6 h. The absolute bioavailability of the orally administered compound was found to be 14%, which was a remarkable improvement compared to zero-to-low oral bioavailability of the native peptide. Compound **1** was effective in stimulating LH release at 20 mg/kg after oral administration. The method was validated at a linear range of 0.01–20.0 µg/ml and a correlation coefficient of r^2 ≥0.999. The accuracy and precision values showed the reliability and reproducibility of the method for evaluation of the pharmacokinetic parameters. These findings showed that the lactose derivative of LHRH has a therapeutic potential to be further developed as an orally active therapeutics for the treatment of hormone-dependent diseases.

1. Introduction

LHRH is a neuroendocrine decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$) secreted by hypothalamus in a pulsatile manner, which stimulates its cognate receptor in the pituitary gland to release gonadotropins including luteinising hormone (LH) and follicle-stimulating hormone (FSH). Subsequently, the production of gonadotropins regulates the secretion of sex hormones in both males and females (Clayton and Catt, 1981). Due to the biological importance, various LHRH analogues have been designed and developed after LHRH was sequenced in 1971 (Matsuo et al., 1971; Schally et al., 1971). LHRH analogues are used clinically for the treatment of various hormone dependent diseases including prostate and breast cancers, endometriosis, fertility disorders and precocious puberty (Huirne and Lambalk, 2001). The continuous administration of LHRH or its agonists leads to an initial surge in the release LH, FSH and sex hormones subsequently, followed by down-regulation of the LHRH receptors and suppression of gonadotropin secretion (Millar et al., 2004).

Endogenous LHRH has a short half-life of 4-8 min in human plasma (Barron et al., 1982; Redding et al., 1973). It is also rapidly degraded by organs including liver, kidneys, anterior pituitary, posterior pituitary, and hypothalamus (Müller et al., 1997). Due to poor stability, LHRH is not able to provide a long-term stimulation of the pituitary gland and exert a strong and long-acting agonist effect. Replacement with D-amino acids at cleavage sites of native peptides improves their stability against enzymatic digestion and thereby prolongs the biological half-life. Substitution of Gly[6] with D-amino acid in endogenous LHRH enhances the enzymatic resistance and also receptor binding affinity of the peptide, which leads to a better agonist activity (Coy et al., 1976; Schally AV, 2003). Triptorelin is a synthetic super agonist of LHRH containing D-Trp at position 6 with a longer half-life (19 min) than the native peptide (Barron et al., 1982) and is parenterally administered for prostate cancer treatment (Heyns, 2005).

Although LHRH agonists have shown improved metabolic stability compared to the native peptide, they are still not orally effective. Poor membrane permeability and susceptibility to digestion by gastrointestinal enzymes give rise to a low oral bioavailability of LHRH agonists (less than 1%) (Iqbal et al., 2012). These analogues are distributed into the extracellular space and metabolized by digestive enzymes and kidney. It is believed that LHRH analogues are predominantly cleared by the kidney due to rapid and extensive renal

uptake following IV administration (Handelsman and Swerdloff, 1986). All commercial analogues of LHRH are administered through parenteral routes including subcutaneous and intramuscular (Beyer et al., 2011; Padula, 2005). As the oral route is the preferred route of administration by patients, development of an oral delivery system for therapeutic peptides like LHRH is highly desirable. Manipulation of peptide structures using glycosylation strategy is known to be an effective approach to improve the metabolic stability and membrane permeability of modified analogues (Christie et al., 2014; Moradi et al., 2014; Powell et al., 1993). Glycosylation has been shown to improve the efficacy of some peptides *in vitro* and *in vivo* (Egleton et al., 2000; Wu et al., 2006; Yamamoto et al., 2009).

Liquid chromatography/mass spectrometry (LC/MS) is a highly selective automated tool for quantitative sample analysis (Niessen, 2003). In addition to high sensitivity and selectivity, LC/MS has the advantage of providing data that are easy to interpret compared to other applicable techniques (Gillespie and Winger, 2011). Immunoassays are other routine techniques used for screening and quantification of peptides. However, these assays have some drawbacks such as higher incidence of false positive results and the requirement for specific antibodies. Therefore, developing an LC/MS-based method can provide highly accurate and reliable peptide quantification in biological samples (Sofianos et al., 2008).

In our previous study, we showed that conjugation of glycosyl units to LHRH peptide enhanced the *in vitro* stability and permeability of the modified peptides across Caco-2 cell monolayers (as an intestinal model). Among all glycosylated analogues, lactose conjugated LHRH demonstrated the best *in vitro* stability and membrane permeability (Moradi et al., 2013). In the current study, we measured the bioavailability and pharmacokinetic parameters of the lactose-modified LHRH as the lead compound in rats. An LC/MS-based method was developed for quantitative sample analysis followed by compound extraction from serum using an optimized method of extraction. The stimulatory effect of the orally administered compound on LH release in rats was also examined over 24 hours.

2. Experimental

2.1 Materials and apparatus

Lactose[Q^1][w^6]LHRH (compound **1**) and lactose[Q^1]LHRH were synthesized according to the published methods (Moradi et al., 2013). Lactose[Q^1]LHRH was used as the internal standard (IS). HPLC-grade acetonitrile (MeCN) was purchased from RCI Labscan Ltd. (Bangkok, Thailand). Methanol (MeOH) in HPLC grade was purchased from Merck

biosciences (VIC, Australia) and Formic acid (analytical grade, 99%) from Univar, Australia. Phosphate buffer saline (PBS) was purchased from Invitrogen Life Technologies.

2.2 Animals

Male Sprague Dawley rats weighing between 140–170 g were purchased from UQ Animal Resource Centre (ARC) and were kept for one week acclimatization period prior to initiation of experimental procedures. Rats were housed in groups of two or three with *ad libitum* access to food and water, in a room with controlled temperature (22.2 ± 0.2 °C) and humidity (51–65%) on a photo period of 12 h light/12 h dark. For experiments, rats were divided into 3 groups of 5 animals; negative control (PBS-treated group), intravenously (IV) and orally (PO) dosed groups. All experimental procedures were approved by The University of Queensland Animal Ethics Committee (AEC#SCMB/005/11/ARC).

2.3 Sample preparation

The rat plasma samples were thawed at room temperature and spiked with 100 µl of IS solution (to a final concentration of 1 µg/ml). Analytes were extracted from plasma using a liquid-liquid extraction method. Different concentrations of acidified MeCN (95%, 80% and 60%) and MeOH (95%, 80% and 60%) with 0.1% formic acid were used to optimize the analyte extraction method. Plasma proteins were precipitated by the addition of 500 µl extraction solutions and were then vortexed and centrifuged at $14000 \times g$, 15 min. The supernatant was collected and evaporated under a slow stream of nitrogen gas using a five-valve glass manifold. Extracted samples were reconstituted in 40 µl of water-acetonitrile-formic acid (90:10:0.1) and injected into the LC/MS system for analysis.

2.4 Preparation of calibration standards and quality control samples

Stock solutions of compound **1** (100 µg/ml) and IS (10 µg/ml) were prepared in water: acetonitrile 90:10 containing 0.1% formic acid. Serial dilutions of the compound (60-0.2 µg/ml) were then prepared from the stock solution to be used as calibration samples. Calibration standards were freshly prepared by spiking dilutions into blank rat plasma to yield final plasma concentrations ranging from 0.05 to 30.0 µg/ml of compound **1** and 1µg/ml of IS solution. Quality control (QC) samples at the lower limit of quantification (LLOQ), low, middle and high concentrations (0.01, 2, 10 and 20 µg/ml) were made daily from separately prepared stock solutions. The selected dilutions were added to 100 µL of plasma spiked with 20 µl of IS solution at a final concentration of 1 µg/ml). Similar extraction

method was then used for the preparation of QC samples. Calibration curves were plotted as the peak area ratio (Compound 1:IS) vs concentration.

2.5 LC–MS analysis and quantification

LC/MS was carried out on a Shimadzu HPLC (LC-10AT) system coupled to a PE Sciex (AB Sciex) API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Concord, Ontario, Canada). The instrument was operated in positive ion mode under the following conditions: Ion-Spray voltage, 5000 V; source temperature, 550 °C; curtain gas (nitrogen) at 8; collision gas (nitrogen) at 5; declustring potential at 50; focusing potential at 220 and entrance potential at 10. Chromatographic separation was performed on a Phenomenex luna C18 column (5 μm, 50 mm × 2.0 mm) with a gradient mobile phase of solvent A (0.01% acetic acid in water) and solvent B (90% acetonitrile, 10% water and 0.01% acetic acid). The compound was eluted with a 20-35% solvent B over 5 min at a flow rate of 0.5 ml/min. Total injection volume was 15 μL. The extracted ion chromatogram with m/z 877.1 at 3.3–3.4 min and m/z 812.2 at 3.9–4.1 min was detected for compound **1** and IS, respectively.

2.6 Method validation

The accuracy and precision, calibration curve performance and recovery of the method were evaluated. The calibration curve was plotted in the range of 0.05-20 μg/ml using 1/x weighting regression model. Calibration curves were evaluated using three separately prepared batches. For testing the accuracy and precision of the method, intra- and inter-day assays were performed for all QC samples. The intra-day assay was performed within one day by analysing triplicate of each concentration of QC samples. The inter-day assay was carried out on four separate days for QC samples (each concentration in triplicate) and repeated twice (in two separate weeks). The recovery efficiency of the extraction procedure was performed at three concentrations of compound **1** (0.01, 10, and 20.0 μg/ml) and IS (1 μg/ml) from rat plasma.

2.7 *In vivo* pharmacokinetic study

Rats were administered compound **1** at 2.5 mg/kg IV, 10 and 20 mg/kg PO. About 300 μL of blood sample was collected from each rat by tail bleed prior to the start of the experiment and at selected time points (0.5, 1, 2, 4, 6, 8, 12 and 24 h). Samples were allowed to clot for 2 h at room temperature, followed by centrifugation at 1000×g for 20 min. Serum aliquots were stored at −80°C.

2.8 LH release assay

Serum concentration of LH was measured by a sandwich ELISA, strictly adhering to methodology as published previously (Steyn et al., 2013). Briefly, a 96-well high-affinity binding microplate was coated with monoclonal antibody (anti-bovine LH beta subunit) and incubated overnight at 4°C. A standard curve was generated using a 2-fold serial dilution of mouse LH in 0.2% (w/v) BSA-1×PBS-T (PBS with 0.05% Tween 20). The LH standards and plasma samples were incubated with 50 µL of detection antibody (polyclonal antibody, rabbit LH antiserum) for 1.5 h followed by the addition of 50 µL horseradish peroxidase-conjugated antibody (polyclonal goat anti-rabbit antibody) and 1.5 h incubation at room temperature. O-phenylenediamine, substrate containing 0.1% H_2O_2 was added to each well and left at RT for 30 minutes. The reaction was stopped using 3 M Hydrochloric acid. The absorbance of each well was read at a wavelength of 490 nm (Sunrise; Tecan Group). The concentration of LH in whole blood samples was determined by interpolating the OD values of unknowns against a nonlinear regression of the LH standard curve. LH secretory responses were expressed as the area under the curve (AUC) after normalizing data to the baseline value. The within and between assay coefficient of variation of LH assays were below 5%.

2.9 Data analysis and statistical evaluation

The pharmacokinetic profiles, including area under the plasma concentration *vs.* time curve (AUC), half-life ($t_{1/2}$) and clearance (Cl) of each rat were analyzed by non-compartmental analysis (Phoenix WinNonlin 1.2; Certara Inc., Princeton, NJ, USA). The maximum plasma concentration (C_{max}) and the time the C_{max} is reached (T_{max}) were directly computed from the plasma concentration *vs.* time graph. The oral bioavailability (F%) was calculated by the following equation:

$$F\% = 100 \times \frac{Dose\ IV \times AUC\ PO}{Dose\ PO \times AUC\ IV}$$

All the data are expressed as mean ± standard deviation. Statistical analysis of pharmacokinetic parameters was calculated using ANOVA followed by the Dunnett's post hoc test.

3. Results

3.1 Method validation and quantification of compound 1 in rat plasma

The chemical structures of compound **1** and compound **2** (IS) are shown in Fig. 1. Lactose[Q^1]LHRH was used as IS due to the structural similarity to compound **1.** The extraction of compound from rat plasma was performed using different concentrations of acidified MeOH and MeCN solutions. Among all extraction solutions tested, the best recovery of compound **1** and IS from rat plasma was obtained using 95% of MeCN in water. The recovery (%) was found to be above 80% for 0.05, 2 and 10 µg/ml of compound **1** (83.7%, 89.8%, and 91.3%, respectively).

The calibration curve showed an acceptable linearity at r^2=0.999 for the range of concentration used, from 0.05 to 20 µg/ml (Fig. S1). The lower limit of quantification (LLOQ) was 0.01 µg/ml. The reproducibility of the method was confirmed by intra- and inter-day assays which were determined by analysing the LLOQ (0.01 µg/ml), low QC (2 µg/ml, n=3), medium QC (10 µg/ml, n=3) and high QC (20 µg/ml, n=3) on four separate runs. The inter-day precision did not exceed 12% for the four concentrations of QC samples and the intra-day precision of the assay was between 2% and 15%. The accuracy of intra- and inter-day assays ranged from 95% to 105% for the analyte and IS (Table S. I).

The method selectivity was determined by analysing the plasma samples spiked with the highest concentration of compound **1** without IS. No signal was detected for IS showing that there was no interference of the IS signal with the compound's peak. Analysis of four sources of blank plasma prepared from different rats showed no signal for compound **1** and IS indicating that source of plasma did not affect the signals obtained from compound **1** and IS.

3.2 Pharmacokinetic studies

Pharmacokinetic parameters were analysed by non-compartmental analysis. The results are summarised in Table I as mean±SD of measurements from five animals per group. Following IV and PO administrations, the plasma concentration of the compound at various time points (during 24 h) was measured using the established LC/MS method (Fig. 2). The AUCs were

obtained at 1.27±0.76 µg/ml*h and 0.709±0.24 µg/ml*h for IV and PO doses, respectively. The mean volume of distribution (V_d) and clearance of compound **1** were found to be 36.49 ml/kg and 1.96±0.52 ml/h/kg, respectively, after IV administration. The peak plasma concentration of the compound was achieved after 2 h (C_{max}= 0.11±0.03 µg/ml). The half-life of compound **1** in plasma was 2.6 h after PO dose, which was a significant improvement compared to the short half-life of native LHRH as reported in literature (4 min) (Redding et al., 1973). The oral bioavailability of the compound was measured to be 14%.

Table 1: Pharmacokinetic parameters of compound **1** in rats after administration at 2.5 mg/kg IV and 10 mg/kg PO. Data are presented as mean ± SD (n = 5).

Parameters	Value (Mean+SD) IV	Value (Mean+SD) PO
Body weight (g)	171±25.08	164.4±18.40
$AUC_{0-\infty}$ (µg/ml*h)	1.27±0.76	0.709±0.24
$T_{1/2}$ (h)	2.9±0.63	2.6±0.84
Cl (ml/h/kg)	1.96±0.52	-
V_d (ml/kg)	36.49	-
C_{max} (µg/ml)	-	0.11±0.03
T_{max} (h)	-	2
F (%)	-	14

$T_{1/2}$: half-life; Cl: clearance; V_d: volume of distribution; C_{max}: maximum concentration; T_{max}: time to reach C_{max}.

3.3 Efficacy of compound 1 in the release of LH

To determine the changes in LH level after oral administration of compound **1,** the normalised area under curves (nAUC) of LH released was obtained over 24 h. A marked increase in the level of LH was observed after oral administration of 20 mg/kg of compound **1** compared to the control (PBS) group (from nAUC=4.45± 1.028 ng/24h in control group to

nAUC=11.33± 1.65 ng/24h). However, the oral dose of 10 mg/kg did not stimulate the release of LH significantly over 24 h (Fig. 3).

4. Discussion

In the present study, the pharmacological properties of lactose-[Q^1][w^6]LHRH was evaluated after PO and IV administration to rats. Due to the poor oral bioavailability, all commercial derivatives of LHRH are administered parenterally which is inconvenient for patients. The main objective of this research was to improve the bioavailability of LHRH following oral administration. Modification of therapeutic peptides by substitution of D-isoform of amino acids is known to be the effective strategy to improve their pharmacological properties and metabolic stability (Seitz, 2000; Werle and Bernkop-Schnürch, 2006). Glycosylation is another useful approach to improve the metabolic stability of peptides in physiological environments and increase their biological activity (Simerska et al., 2009; Ueda et al., 2009). We applied D-amino acid substitution together with glycosylation strategy to address the associated challenges and enhance the bioavailability of LHRH peptide. In our preliminary *in vitro* studies, we showed that glycosylation significantly enhanced the metabolic stability (up to 4-fold) and apparent permeability (7 to 15-fold) of LHRH across intestinal cell membranes (Moradi et al., 2013; Moradi et al., 2014). Based on those results, we selected compound **1** as the most promising LHRH glycosylated derivative for oral administration and investigated the pharmacological profile of this analogue followed by oral administration to rats.

For pharmacokinetic evaluation of compound **1,** an accurate analytical tool was required to determine the peptide's plasma concentration. Therefore, an LC/MS method was developed and validated for quantitation purposes. LC/MS is an automated technique with broad applications in quantification of pharmaceutical compounds and their metabolites in biological matrices. The selectivity, sensitivity and cost effectiveness of MS based methods make them a preferred analytical technique in pharmaceutical industry (Lee, 2003). The developed method in this study was capable of detecting the compound over a range of 0.01 to 20 µg/ml with a detection limit of 0.01 µg/ml. The precision and accuracy of the method was assessed by performing intra- and inter-day assays The precision value obtained was below 12% for the four QC levels which was in acceptable limit based on FDA guidelines (the precision value should not exceed 15%) (Rower et al., 2010). The high accuracy (98.2% and 97.9% for intra-day and inter-day, respectively) was also obtained showing the reliability of the developed method. Consequently, the validation of the method showed good

reproducibility, accuracy, precision and linearity for quantification of compound **1** in rat plasma.

A quick and easy extraction protocol was performed for precipitation of plasma proteins using liquid-liquid extraction method. It yielded over 80% recovery for three selected concentration of the compound. The high and consistent extraction recovery showed a negligible loss of the analyte during the sample preparation process, and did not vary from sample to sample.

A significant improvement in the bioavailability (14%) and half-life of compound **1** (2.6 h) was observed after oral administration of compound **1** compared to the endogenous peptide (Iqbal et al., 2012; Redding et al., 1973). The increased half-life of the compound may account for the enhancement of its bioavailability. These findings demonstrate that the attachment of lactose to LHRH analogue significantly improved the pharmacological properties of native LHRH. This is in agreement with other studies we previously published showing that the modification of opioid peptide endomorphin-1 by a lactose moiety resulted in significant analgesic activity of the peptide following oral administration to rats (Varamini et al., 2012). To the best of our knowledge, this is the first report of an orally administered LHRH analogue with a remarkable half-life and bioavailability.

Acute administration of LHRH agonists stimulates the pituitary gland to release LH, whereas the chronic administration results in suppression of the pituitary-gonadal axis and blockade of LH secretion (Pinski et al., 1996). Following single-dose oral administration (acute administration) of compound **1** to rats, their plasma LH level increased significantly compared to the negative control group. This finding demonstrated that compound **1** was able to produce stimulatory effect *in vivo* following acute administration. Taken together, we improved the oral bioavailability of LHRH by conjugation of a lactose moiety and D-Tyr[6] substitution while maintaining the activity.

5. Conclusions

In the present study, we applied glycosylation strategy together with D-amino acid substitution to develop an orally active LHRH analogue. A sensitive and specific LC/MS quantification method was developed and validated to evaluate the pharmacological activity of lactose-[Q[1]][w[6]]LHRH *in vivo*. The method was reproducible and reliable to be used for quantitative analysis of the compound in rat plasma. We showed that the conjugation of lactose residue to LHRH peptide increased its half-life and oral bioavailability in rats significantly. The newly designed analogue could also stimulate LH secretion upon oral

administration. The improved pharmacological properties of lactose-$[Q^1][w^6]$LHRH render this analogue a promising candidate towards the development of an orally available LHRH therapeutics.

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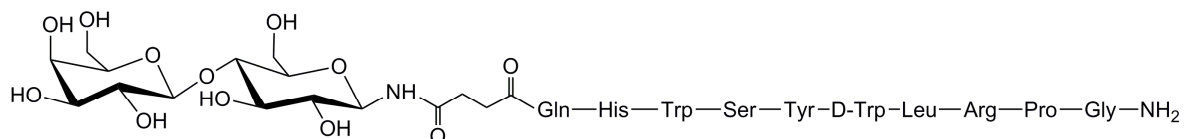
Figure captions

Figure 1: Chemical structures of (**A**) lactose [Q^1][w^6]LHRH (compound **1**) and (**B**) lactose [Q^1]LHRH (Compound **2**; IS).

Figure 2: Plasma levels of compound **1** (mean ± SD, n=5) following 2.5 mg/kg IV bolus (A) and 10 mg/kg oral gavage (B) in intact male rats measured by LC/MS.

Figure 3: Changes in the plasma level of LH calculated from a nAUC over 24 h following oral administration of compound **1** at 10 and 20 mg/kg**.** Each column represents the Mean ± SD (n=5). Statistical analysis was performed using a one-way ANOVA followed by the Dunnett's post hoc test and compared to PBS group (***, p<0.01).

Fig. 1

(A)

(B)
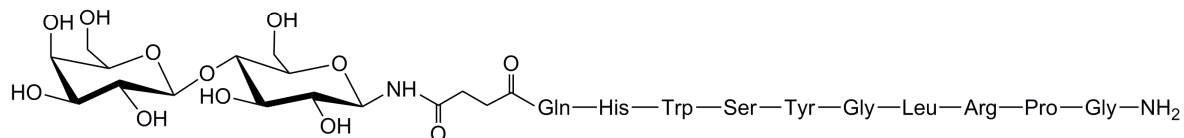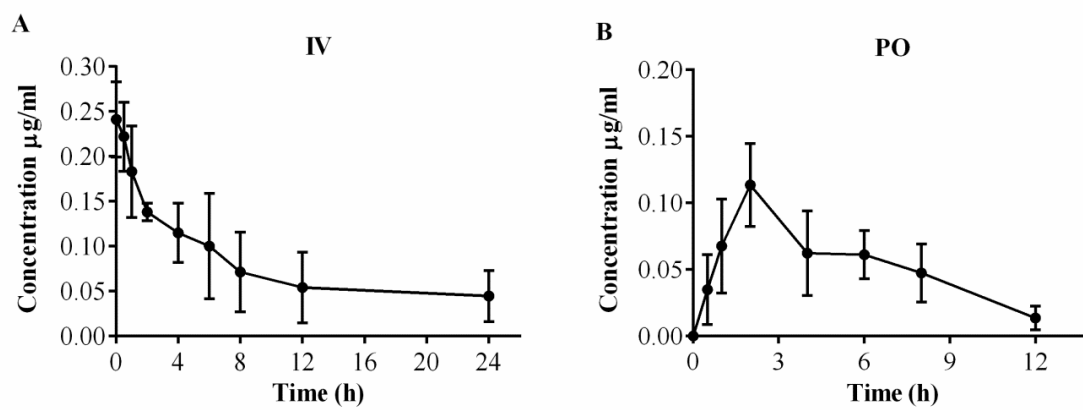

Fig. 2

Fig. 3