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In vivo evaluation of the safety of triptolide-loaded hydrogel-thickened microemulsion

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ABSTRACT

Triptolide (TP) is a major active component of *Tripterygium wilfordii* Hook F (TWHF), which is used to treat rheumatoid arthritis (RA). TP has a narrow therapeutic window. To increase the therapeutic index of TP, a novel TP-loaded transdermal delivery system, named TP-loaded hydrogel-thickened microemulsion (TP-MTH), has been developed to treat RA. Our previous studies have demonstrated the good efficacy of TP-MTH in animals. This paper evaluated the safety of TP-MTH with several animals. Results demonstrated no obvious toxicities in a series of toxicity tests: acute toxicity study of TP-MTH (1.2 mg/kg) in rabbits, 6-month long-term toxicity study of TP-MTH (0.06, 0.18, 0.54 mg/kg) in rabbits, safety pharmacology study of TP-MTH (0.03, 0.09, 0.27 mg/kg, for 5 d) in mice and beagle dogs, skin irritation study in rabbits, and skin allergic reaction test in guinea pigs. Only mild reversible skin irritation signs were observed on the skin of animals. These studies suggest that topical TP-MTH is a promising drug formulation for the treatment of RA.

1. Introduction

Rheumatoid arthritis (RA) is a systemic disease associated with symptoms of redness, swelling, fever, and pain in local joints and surrounding tissues. It is one of the most common inflammatory conditions, and it is also a major cause of disability in developing countries (Hannington-Kiff, 1990). Many drugs (analgesics, non-steroidal or steroidal anti-inflammatory agents, and disease-modifying anti-rheumatic drugs) have been developed to treat RA over the past few decades. However, severe side effects, low efficacy, and bad patient compliance for these drugs have driven efforts to seek more effective and safer therapeutic agents that can be used for long-term administration (Badger and Lee, 1997).

Tripterygium wilfordii Hook F (TWHF) is a vine-like plant that grows in southern China. It has been used to treat leprosy and RA since several years; however, its efficacy has been recognized by modern medicine only recently (Chen, 2001). Triptolide (TP), a major active component isolated from TWHF, whose chemical structure is shown in Fig. 1, is a diterpenoid triepoxide that shows multiple pharmacological activities, such as anti-inflammatory, immuno-suppressive, male anti-fertility, and anti-tumour activities (Brinker et al., 2007). Two extracts of TWHF from methanol/chloroform and ethyl acetate have been reported to be effective in the treatment of patients with RA generally by the oral route. TP was deemed to account for the anti-inflammatory and immuno-suppressive activities of the extracts (Tao et al., 1989; Tao and Lipsky, 2000). However, TP was not widely used clinically due to its water insolubility and severe toxicities toward the gastrointestinal, renal, cardiac, hepatic, hematopoietic, and reproductive systems (Shamon et al., 1997; Sun et al., 2001). In addition, TP is a special drug with a narrow therapeutic window, of which the effective dose is nearly equal to its toxic dose (Zheng, 1991). To overcome these drawbacks, TP and its analogues have been modified to reduce toxicity by chemical modification and biotransformation methods in recent years (Yu et al., 1992; Fidler et al., 2003; Ning et al., 2003; Zhou et al., 2005; Aoyagi et al., 2006).

One of the efficient ways to reduce toxicity is to deliver the drug to the desired site of action in the body to decrease or avoid the side effect at a non-target site. The controlled release delivery system and targeted drug delivery system have been used to deliver TP to reduce its toxicities (Xiong et al., 2005; Zheng et al., 2006). Our laboratory developed systems of TP-loaded in solid lipid nanoparticle (TP-SLN), ploy (D,L-lactic acid) nanoparticle (TP-PLA-NP), and microemulsion (TP-ME) (Mei et al., 2003; Chen et al., 2004; Liu et al., 2004). Our previous studies demonstrated that the system of TP-loaded in nanoparticles had less toxicity in the rat liver and reproductive system (Liu et al., 2005; Mei et al., 2005). Among the new delivery systems, the transdermal delivery system (TDDS) is an attractive route for TP administration. The potential advanta-

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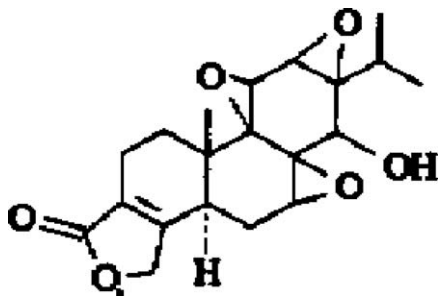

Fig. 1. The chemical structure of triptolide.

ges of TDDS include prevention of first-pass metabolism, elimination of gastrointestinal irritation, low dosing frequency, and rapid termination of drug action (Parikh and Ghosh, 2005). In the transdermal delivery of TP, microemulsion was used to reduce the diffusion barrier of the stratum corneum by acting as a permeation enhancer (Chen et al., 2004; Kogan and Garti, 2006). We previously prepared the novel TP-loaded transdermal delivery system, named TP-loaded hydrogel-thickened microemulsion (TP-MTH), where TP was loaded at an extremely low concentration of 0.003% into MTH to prevent the skin irritation caused by high concentrations of TP; the permeation rate of TP-MTH was 5.8 times greater than that of the control gel in in vitro permeation studies on hairless mouse skin (Chen et al., 2007). Our previous studies have also demonstrated the efficacy of TP-MTH (Xu et al., 2007).

In this paper, the preclinical safety of this novel TP-loaded transdermal delivery system was evaluated with several animals through various studies, including an acute toxicity study, a 6-month long-term toxicity study, safety pharmacology study, skin irritation study, and skin allergic reaction test. The long-term goal of this work is to develop a topical TP formulation for clinical use for increasing the therapeutic index.

2. Materials and methods

2.1. Drugs and reagents

TP (purity > 99%) was supplied by Fujian Institute of Medical Sciences (Fuzhou, China). TP-MTH and MTH were prepared in our laboratory. MTH consisted of 3% isopropyl myristate (IPM), 30% Tween 80, 0.75% Carbomer 940, 15% propylene glycol (PG), 1% triethanolamine (TEA), 2% menthol, and water (Chen et al., 2007). TP was loaded in MTH in concentrations of 0.0015%, 0.003%, 0.006%, 0.009%, 0.012%, 0.024% and 0.027% (10 g MTH containing 0.15, 0.3, 0.6, 0.9, 1.2, 2.4 and 2.7 mg TP, respectively). Dinitrochlorobenzene (DNCB) was obtained from Shanghai Chemical Reagent Corporation (Shanghai, China). Pentobarbital sodium was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals

Kunming mice, New Zealand rabbits and English guinea pigs were obtained from the Laboratory Animal Center, Hubei Academy of Preventive Medicine (Wuhan, China). Beagle dogs were obtained from Guangdong National Beagles Resources Research Center (Guangzhou, China). The animals were housed in a well-ventilated room with a constant temperature of 23 ± 2 °C, relative humidity of 40–70%, and illumination (12 light/dark cycles). All animals were fed with standard animal chow daily and had access to drinking water ad libitum. The protocol of the study was approved by the Ethical Committee of Huazhong University of Science and Technology. Safety evaluation of TP-MTH was performed in compliance with China State Food and Drug Administration's (CSFDA) current Good Laboratory Practice (GLP) regulations and in accordance with CSFDA's relative Safety Evaluation Test Guidelines (2005).

2.3. Acute dermal toxicity study in rabbits

An acute dermal toxicity test was conducted in rabbits to determine the potential toxicity of TP-MTH from a single topical application. Sixteen rabbits (weight, 2.2–2.5 kg) were randomly divided into two equal groups (one test group and one control group). Each group was then subdivided into two subgroups: the intact skin subgroup and the dermal injury subgroup, and each subgroup contained the same number of male and female rabbits. Twenty-four hours before the experiment, the rabbits were depilated, and dermal injury was produced by scraping the skin with a sterilized needle until oozing of blood began to appear under anesthesia with intravenous 3% pentobarbital sodium in the dose of 30 mg/kg. Then, 2 g/kg TP-MTH (0.06%, the dose of TP is 1.2 mg/kg) and 2 g/kg MTH were smeared onto the depilated skin areas (150 cm^2) covering both sides of each rabbit in the two groups, respectively. The day of application was considered Day 0 of the study. After 24 h of exposure to the test substance, the remnants of the drug and vehicle were gently washed away. The mortality, signs of gross toxicity, and behavioral changes after application were observed at least once a day thereafter for 14 d. All rabbits were euthanized by intravenous pentobarbital sodium in the overdose on Day 14. Necropsies were performed on all animals, and the examinations of organs and tissues are listed in Table 1. Heart, liver, spleen, lung, kidneys, brain, thymus, adrenal glands, testes, prostate, seminal vesicles, ovaries and uterus were weighed and expressed as percent of body weight. Then, all the tissues were fixed and preserved in 10% neutral buffered formalin, embedded in paraffin and subjected to hematoxylin–eosin staining. The pathological observations of all tissues were performed on gross and microscopic bases.

2.4. Long-term toxicity study in rabbits

Rabbits (2.2–2.5 kg) were randomly divided into four groups, with four males and four females per group. TP-MTH (2 g/kg) with TP contents of 0.003%, 0.009% and 0.027%, and MTH (2 g/kg) were smeared onto the depilated skin areas (150 cm^2) of rabbits of different groups once a day, six days a week, for six months. Body weight and food consumption were measured and recorded weekly. The general physical condition of each rabbit was observed daily. After three months and six months of the administration, blood samples were collected under anesthesia with intravenous 3% pentobarbital sodium in the dose of 30 mg/kg for hematological and clinical chemistry analyses using a fully automated cell analyzer (Abbott Cell-Dyn 3700, USA) and an automatic biochemical analyzer (Hitachi 7060, Japan), as specified in Table 1. The abovementioned observations were continued for four weeks after the treatment was stopped. Animals were euthanized by intravenous pentobarbital sodium in the overdose at the end of the study, and necropsies were performed on all animals. The examinations of organs and tissues are listed in Table 1. Heart, liver, spleen, lung, kidneys, brain, thymus, adrenal glands, testes, prostate, seminal vesicles, ovaries and uterus were weighed and expressed as percent of body weight. Then, all the tissues were fixed and preserved in 10% neutral buffered formalin. Tissues from the control group and the high-dose group were embedded in paraffin and subjected to hematoxylin–eosin staining. The pathological observations of all tissues were performed on gross and microscopic bases. When lesions were observed in the high-dose group, the affected organs were also examined in the lower-dose group of TP-MTH.

2.5. Safety pharmacology study in mice and beagle dogs

Four experiments were performed in this safety pharmacology study as described previously (Kim and Shin, 2004). In the first experiment, the general behavior of mice was observed. Mice (14–16 g) were randomly divided into five groups, with five males and five females per group. The animals were depilated on the back (1 cm^2). The test groups received 1 g/kg of 0.003%, 0.009% and 0.027% TP-MTH, and the vehicle group was treated with 1 g/kg MTH once a day for 5 d, while the normal

Table 1
Parameters evaluated of male and female rabbits in acute dermal toxicity study and long-term toxicity study.

Hematological and biochemical parameters evaluated
Red blood cells, hematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelets, reticulocytes, white blood cells, neutrophils, eosinophils, lymphocytes, monocytes, thrombin time, total protein, albumin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, glucose, total cholesterol, triglyceride, blood urea nitrogen, creatinine
Organs and tissues examined
Skin, heart, liver, spleen, kidneys, adrenals, mesenteric lymph node, thoracic aorta, salivary gland, sternum, femur, thymus, trachea, lung (including bronchus), thyroids, parathyroids, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, pancreas, urinary bladder, seminal vesicles (including the coagulating glands), prostate, testes, epididymides, ovaries, oviducts, uterus, brain (including cerebrum and cerebellum), pituitary gland, sciatic nerve, skeletal muscle, spinal cord