Toxicological Evaluation of the New Calcium Antagonist VULM 993

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Abstract. The tolerance of the new calcium antagonist VULM 993 was investigated in a series of toxicological studies. The following results were obtained: the maximum tolerated oral dose in acute toxicity was 10,000 mg/kg for mice and 6600 mg/kg for rats, for venous administration it was 26.1 mg/kg in mice and 32.2 mg/kg in rats. In subacute oral toxicity test in rats, VULM 993 showed no toxic effect up to 300 mg/kg/d. The drug was not teratogenic in rats (5, 50 or 250 mg/kg/d, p.o.). VULM 993 did not show any positive response in tests for genotoxicity *in vitro*. Transplacental study of VULM 993 in rabbits indicated active placental barrier function in the late stage of pregnancy. The toxicological profile of VULM 993 is characterised by a high tolerance in all relevant species of experimental animals, and no biologically significant mutagenic potential was recorded.

Key words: 1,4-dyhydropyridine — Toxicity – Genotoxicity — Placental transfer

Introduction

VULM 993, 4-(3-chlorophenyl)-3-etoxycarbonyl-5-(1-(1,4dioxaspiro[4.4]nonan-2-yl) methylenoxycarbonyl)-2,6-dimethyl-1,4-dihydropyridine, is a new calcium antagonist with potent antihypertensive activity (Ježek and Kochan 1997).

Toxicological studies of VULM 993 (acute, subacute, reproductive toxicity, genotoxicity and placental transfer) are presented in this paper. The tests were selected and conducted in accordance with the legislative guidelines and OECD recommendations as a part of a larger safety evaluation programme of this drug.

Materials and Methods

Animals

Test animals (SPF Wistar rats , SPF ICR mice) were purchased from Velaz Prague, Czech Republic, and New Zealand white female rabbits from Interloco, Očkov, Slovakia.

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Rats and mice were housed in plastic cages, rabbits in stainless steel cages The animals were fed commercially available food and were allowed free access to tap water Room temperature and relative humidity were set at controlled values (rats and mice 25 ± 3 °C and 60 ± 20%, rabbits 20 ± 2 °C and 40–70%) Artificial illumination was provided between 7 00 and 19 00 h

Tests

1 Acute toxicity
Acute toxicity of VULM 993 in mice, rats and rabbits was assessed using different routes of administration The test substance was administered orally, dissolved in suspension of 1% carboxymethylcellulose (CMC), and intravenously in 12% Cremophor EL (BASF, Germany)

The tests were performed in accordance with the OECD Guideline 401 (1987)

2 Subacute toxicity
A 28-day toxicity test of orally administered VULM 993 was performed in Wistar rats The animals ($n = 120$) were divided into four groups The dose levels (selected on the basis of acute toxicity and daily therapeutic dose) of 0, 30, 100 and 300 mg/kg were tested in both sexes VULM 993 suspended in 1% CMC was administered orally by gavage in a volume of 5ml/kg/d The experimental design of the study is given in Table 1 Clinical signs and mortality were recorded daily Haematology and clinical biochemistry examinations were made using blood collected before dosing, at the end of the administration period, and 2 weeks after the treatment At the end of the experimental period, and during the study when animals were found dead, the main organs of all rats, treated and controls, were weighed and examined histologically All these examinations were performed in accordance with the OECD Guideline 407 (1981)

Table 1. Experimental design of subchronic toxicity study

Species	No/sex/group	Oral dose (mg/kg)	Recovery (2 weeks)
Rat	20	0	10
	10	30	–
	10	100	–
	20	300	10

3 Teratogenicity
The study was conducted according to the current OECD Guideline 414 (1981) Wistar rats were treated orally daily from gestation day 6 through day 15 with doses of 5, 50, and 250 mg/kg/d Behaviour, clinical signs and body weight were recorded throughout the experiment On day 20 of gestation, the dams were sacrificed and gross pathology examination was performed The following parameters were recorded number of corpora lutea, implantations, early and late resorptions, number and sex of viable and dead foetuses, individual foetal and placental weight The live foetuses were examined for external, visceral and skeletal abnormalities (Manson and Kang 1989)

4 Genotoxicity
The aim of this part of the toxicological evaluation was to investigate the possible genotoxic effects of VULM 993 by means of the following tests reverse mutation test using

Salmonella typhimurium (gene mutation in bacteria), point mutation test in V 79 hamster cells (gene mutation in mammalian cells), and cytogenetic test *in vitro* (chromosome damage in mammalian cells) Standard methods were applied in all the tests conducted to determine the mutagenic potential of VULM 993 The *in vitro* assays were carried out both without and with rat liver microsomes, induced by 20-methylcholanthrene as metabolic activator Some of the methodological parameters of the tests are given in Table 2 The reverse mutation test – direct plate assay was performed according to the technique described by Ames et al (1975)

Table 2. Experimental design of mutagenicity tests

Test	OECD guideline	Experimental system		VULM993 concentration
		species	strains	
Gene mutation in prokaryotic cells	471	Salmonella typhimurium	TA 100 TA 98 TA 97 TA 102	1–1000 μg/ plate S9 rat liver induced by 20-methylcholanthrene
Gene mutation in eukaryotic cells	476	Chinese hamster lung	V 79/4	20–60 μg/ml
Chromosome aberrations	473	Chinese hamster lung	V 79/4	0 5–4 μg/ml S9 rat liver induced by 20-methylcholanthrene

In V 79 cells, cytotoxicity of VULM 993 was assessed prior to the proper mutagenicity and clastogenicity tests Gene mutation test in eukaryotic cells was performed by the method of Abbondandolo (1975) The *in vitro* cytogenetic test was conducted according to the method described by Ishidate (1988)

5 Placental transfer

VULM 993 was investigated for its placental transfer and organ distribution in rabbits on day 27of gestation ^{14}C-VULM 993 was administered by oral gavage as a suspension in 1% CMC (5ml/kg) in the dose of 60 mg/kg Control animals (nonpregnant) received only the vehicle at the same dosage volume

The concentrations of ^{14}C-VULM 993 and of its metabolites were determined by radioanalytical methods in maternal and foetal plasma and organs 0 5–6 0 h after administration

6 Statistical analysis

All measured values were analyzed statistically using Barlett test, Kruskal-Wallis test, Fisher test, Student's *t*-test or ANOVA

Results

Acute toxicity

The mean lethal dose (LD_{50}) values are shown in Table 3. LD_{50} values were determinable in all cases for the parenteral route. After oral administration, acute toxicity of VULM 993 was not appreciable in mice and rats. Even the highest dose of 6600 mg/kg in rats and 10,000 mg/kg in mice did not induce any pathological changes.

Table 3. Toxicity of VULM 993 after single oral dose

species	route of administration	LD_{50} (mg/kg) male
mouse	oral	> 10,000
rat	oral	> 6600
mouse	i.v.	26.1 (22.8–30.0)
rat	i.v.	32.2 (28.4–36.5)

Subacute toxicity

No toxic effects of VULM 993 were observed in any of the groups tested and at any time during the study. Male animals receiving the medium (100 mg/kg) and the highest (300 mg/kg) doses showed significantly reduced body weight after two weeks of administration. VULM 993 did not show toxic effects when administered at doses of up to 300 mg/kg for 4 weeks. Behaviour, body weight gain, haematology, clinical biochemistry, organ weight analysis, macro-microscopic examinations revealed no substance-related influence.

Teratogenicity

The drug tested did not induce any toxic effect on the dams. Treatment with VULM 993 did not adversely influence the numbers of implantations, pre-, and post-implantation losses, live foetuses, sex ratio and foetal and placental weight. External, skeletal and internal examinations of the foetuses revealed no evidence of teratogenesis (Ujházy et al. (1996, 1997).

Genotoxicity

The results of the specific tests for gene mutation are summarised in Tables 4 and 5. In the bacterial reverse mutation assay, VULM 993 was tested up to a maximum concentration of 1000 μg/plate in three independent experiments both in the presence and absence of S-9 mix. No substantial increases in the number of revertant colonies were observed in any of the four *Salmonella typhimurium* strains. In the mammalian cell mutation assay, VULM 993 did not increase the incidence of mutant colonies per 10^5 survivor cells. VULM 993 had no mutagenic

Table 4. Evaluation of mutagenicity of VULM 993 in reverse mutation test

Compounds and concentrations (μg/plate)	S9 Mix	number of revertants ± S D / plate			
		TA 100	TA 98	TA 97	TA 102
Control (DMSO)	–	137 ± 9	25 ± 5	138 ± 13	298 ± 36
VULM 993	–				
1	–	149 ± 18	32 ± 5	129 ± 70	268 ± 14
10	–	120 ± 16	26 ± 2	136 ± 40	255 ± 80
100	–	130 ± 14	28 ± 5	144 ± 80	219 ± 10
500	–	140 ± 11	17 ± 5	143 ± 10	206 ± 18
1000	–	122 ± 18	24 ± 4	129 ± 90	248 ± 20
Positive controls*	–	1614 ± 298	320 ± 18	716 ± 6	575 ± 118
Control (DMSO)	+	147 ± 18	35 ± 6	182 ± 29	348 ± 35
VULM 993					
1	+	141 ± 19	38 ± 8	171 ± 26	306 ± 27
10	+	144 ± 10	38 ± 2	163 ± 27	442 ± 31
100	+	158 ± 22	35 ± 8	161 ± 12	405 ± 28
500	+	138 ± 33	37 ± 7	138 ± 15	387 ± 19
1000	+	121 ± 23	29 ± 8	121 ± 15	370 ± 33
Positive controls**	+	560 ± 134	147 ± 31	429 ± 85	457 ± 38

*TA100,TA98, TA102 4-nitroquinoline – N-oxide 20 μg/plate, TA 97 4-nitrophenylenediamine 20 μg/plate, **Benzo(a)pyrene 5 μg/plate

effects in the reverse mutation test and in the HPRT point mutation test. In the cytogenetic study *in vitro*, no significant or dose-dependent increases were observed in the incidence of chromosomal aberration in V 79 cells (gaps, breaks, exchanges), either in the presence or absence of the metabolic activation system, after 24 h treatment

Placental transfer

There were significant differences in the pharmacokinetics of VULM 993 in the plasma between pregnant and nonpregnant rabbits (Fig. 1). VULM 993 was rapidly absorbed by both, reaching C_{max} = 176.3 and 282.1 ng/ml at T_{max} 95 and 122 min, respectively. Elimination half-life was 315 and 266 min, area under the curve (AUC_{0-360}) 90 4 and 126.2 μg/ml/min, respectively. The lower levels of total radioactivity at 120 min in organs of pregnant rabbits, except lungs and livers, corresponded to lower plasma concentrations. No accumulation of the compound and its metabolites were found in maternal organs. The radioactivity measured in foetal organs reached 20 times lower values than in maternal organs

Table 5. Evaluation of the mutagenic potential of VULM 993 in the V79/HPRT assay

Compound	Concentration (μg/ml)	Survival (%)	Mutant frequency/ 10^5 viable cells
Control (DMSO)		72 2 ± 8 9	0 32 ± 0 33
VUM 993			
	20	62 5 ± 16 7	1 14 ± 0 54
	40	42 4 ± 15 2	1 36 ± 1 79
	50	32 0 ± 15 5	0 58 ± 0 49
	60	21 2 ± 4 5	0 19 ± 0 19
EMS	400	73 6 ± 18 8	16 4 ± 6 66

EMS – ethylmethane sulphonate

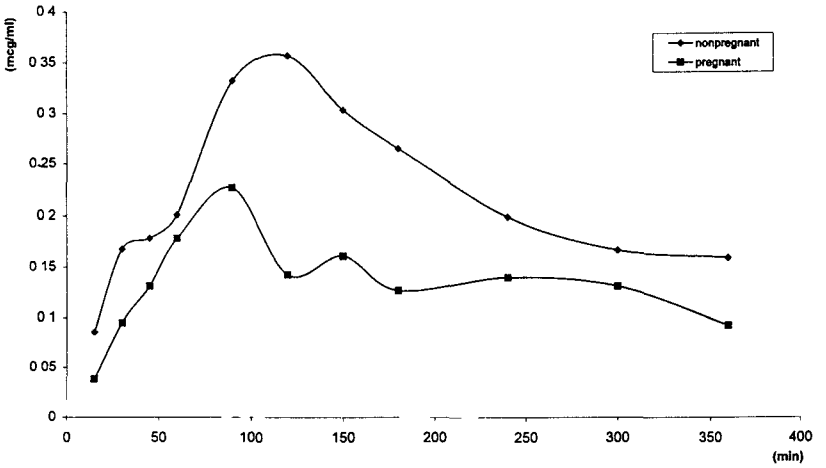

Figure 1. Maternal plasma levels of VULM 993 after oral dosing of 60 mg/kg to rabbits

Discussion

According to the results of the presented toxicological studies, VULM 993 appears to be a safe drug. Its acute toxicity in mice and rats was found to be very low after oral administration (>10,000 mg/kg and >6600 mg/kg, respectively.). After oral administration of VULM 993, the level of LD_{50} was similar to that of nifedipine in rats and higher than after intravenous injection of nicardipine in mice and rats (Gomi et al. 1985). Schlüter (1986) reported LD_{50} values of 3362 mg/kg for nimodipine administered orally in mice, and 16 mg/kg after intravenous administration in rats. Thus VULM 993 may be considered to have low toxicity. In subacute toxicity, VULM 993 showed no toxic effects up to doses of 300 mg/kg on oral administration to rats. Administered orally during organogenesis to pregnant rats in doses of up to 250 mg/kg/d, VULM 993 showed no evidence of teratogenicity.

In three *in vitro* toxicity tests, VULM 993 showed no mutagenic or clastogenic potential. Relatively few data have been reported on mutagenicity of 1,4-dihydropyridines. Obaseki and Coker (1988) and Schlüter (1986) published results of mutagenicity tests in which nifedipine and nimodipine were examined in Ames test, micronucleus test and dominant lethal assays. In these reports no genotoxicity of either of the drugs was found.

In our experiment, the pregnant organism showed higher elimination ability after oral administration of VULM 993, manifested in lower concentrations of the compound and its metabolites in the plasma and organs compared to nonpregnant animals. The low total radioactivity determined in foetuses and foetal organs (<0.07% of dose) was indicative of active placental barrier function on day 27 of pregnancy

In conclusion, the toxicological profile of VULM 993 was characterised by a high tolerance in all species of experimental animals studied as well as in *in vitro* systems.

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