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Evaluation of the irritating influence of carane derivatives and their antioxidant properties in a deoxyribose degradation test

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Abstract:
Previous studies of the propranolol monoterpene derivative (–)-4-[2-hydroxy-3-(N-isopropylamino)-propoxyimino]-*cis*-carane hydrochloride (KP-23) and its diastereoisomers, KP-23R and KP-23S, demonstrated different effects on the cyclic AMP generating system as well as anti-inflammatory, analgesic, antihistaminic and antioxidant activity. The present study examined the influence of KP-23 and its diastereoisomers KP-23R and KP-23S on the skin-irritating activity and the mucous membrane-irritating activity as well as their influence on a late-type contact allergy in the *in vivo* tests. The hydroxyl radical scavenging potential of the three analogues was evaluated using their ability to inhibit Fe(II)/H_2O_2-induced oxidative degradation of 2-deoxyribose (2-DR) in the *in vitro* tests. The results obtained indicated that the hydroxyamine carane derivative did not evoke irritative changes and did not induce a late-type contact allergy in the guinea-pig. Diastereoisomers of KP-23 exhibit antioxidant properties in a dose-dependent manner and protected against OH-radicals generated from the Fenton reaction.

Key words:
carane derivatives, contact allergy, deoxyribose, drug effects, free radical

Introduction

Superoxide anions are precursors of free radicals, which have the potential to react with biological macromolecules and thereby induce tissue damage [9, 26, 33]. Free radicals can adversely alter lipids, proteins and DNA and have been implicated in aging and a number of human diseases. Among reactive oxygen species (ROS), hydroxyl radicals (OH) exhibit the strongest oxidative activity and are widely implicated as the major damaging species in free radical pathology [8, 37]. Cells are protected from free radical-induced damage by a variety of radical scavenging antioxidant proteins, enzymes and endogenous compounds. Antioxidants can neutralize free radicals by acting at different levels, i.e., prevention, interception and repair [28, 39]. Several publications have re-

ported antioxidant activity of drugs from different pharmacological classes [2, 12, 32]. Recent research has suggested that anesthetics, such as propofol, may function as antioxidants, thereby protecting brain tissue from damage, inducing free radicals that protect the lipid components of cell membranes, and protecting cardiac H9c2 cells from hydrogen peroxide induced injury [21, 36].

Allergic contact dermatitis is a form of late type cellular hypersensitivity. It develops after local exposure of the skin (mucous membranes) to low-molecular substances that have an ability to form of the covalent bonds with proteins of the skin. Most bound chemical compounds then become full antigens and induce an increase in the population of lymphocytes T. This reaction can be induced through the endermic application of 2,4-dinitrochlorobenzene (DNCB). The increase in the population of peculiarly sensitive lymphocytes T and the renewed use of DNCB after 14 days from the original application of the allergen causes the sensitized lymphocytes to react as if antigen were present, changing the appearance of the skin within 48 h. This reaction is persistent for some time and later disappears. The repeated contact with the allergenic substance caused that reaction in the skin does not necessarily account for the persistent appearance of the inflammatory and contact dermatitis. This sensitivity to a topical treatment is the most common reason for changes after the usage ointments containing local anesthetics, such as the lidocaine, dibucaine and tetracaine [6, 14, 22]. Patients who are sensitive to these drugs may also react to environmental substances that have homologous chemical construction [29].

In search of new compounds with the local anesthetic activity and better pharmacological effects, derivatives of naturally occurring anesthetic compounds were made. Topical anesthetics are widely used in dentistry, ophthalmology, dermatology, laryngology

Fig. 1. Chemical structures of the compounds investigated

Compounds	Structures	Mol. mass
KP-23R		318.85 g/mol
Bupivacaine		288.43 g/mol
Lidocaine		234.34 g/mol
Tetracaine		264.363 g/mol

and urology, so it is essential that the new preparations did not induce the contact allergic and irritating reactions. One promising compound was KP-23. Compared to previously investigated propranolol analogs containing natural monoterpene structures, the compound (–)-4-[2-hydroxy-3-(N-isopropylamino)-propoxyimino]-*cis*-carane hydrochloride (KP-23), and its diastereoisomers, KP-23R and KP-23S [19], had the most efficient pharmacological activity in the tests used (Fig. 1). Previous studies have revealed that the R- and S-diastereoisomers of KP-23 have different activities in the local anesthetic, anti-aggregating, anti-arrhythmic and anti-spasmolytic tests. The influence of these compounds on the cyclic AMP generating system, and the anti-inflammatory, analgesic, antihistaminic and antioxidant effects of these compounds have been previously described [15–18].

According to the reports of different authors, the terpenes may be the active constituent responsible for the anti-inflammatory activity. It is very promising that a new compound, with potential local anesthetic activity, does not evoke any toxicodermal effects and does not induce any allergic reaction in response to its topical application [6, 29].

In the present work, we investigated the effects of propranolol monoterpene derivatives in the *in vivo* studies on the skin-irritating activity, assessing the late type contact allergy, the mucous membranes-irritating activity and, in an *in vitro* test, the antioxidant activity of KP-23 and its diastereoisomers. The purpose of the present work was to examine the irritating properties of the epimers of KP-23S and KP-23R and the possible toxicoallergic effects on the skin and mucous membranes and to compare them with KP-23.

Materials and Methods

Drugs and reagents

2-Deoxyribose (2-DR), 2,4-dinitrochlorobenzene (DNCB), ascorbic acid, bupivacaine (BUP), ethylenediaminetetraacetic acid (EDTA), lidocaine (LDC), potassium phosphate, tetracaine (TTC), thiobarbituric acid (TBA), and Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were purchased from Sigma-Aldrich, USA. (–)-4-[2-Hydroxy-3-(N-isopropylamino)-propoxyimino]-*cis*-carane hydrochloride and it diastereoisomers (KP-23, KP-23S, KP-23R) were obtained from Institute of Organic Chemistry and Biotechnology, Wrocław University of Technology [19]. Basis – Lipobase creme (Yamanouchi Europe B.V.) was purchased from Cefarm-Kraków S.A. All the other reagents were of standard analytical grade.

PROCEDURES *IN VIVO*

Animals

The studies were carried out using male guinea pigs (300–350 g body weight) and male rabbits (2.0–3.5 kg body weight). The animals were housed in wire mesh cages at a constant temperature (20 ± 2°C) and 12/12 h light-dark cycle. The animals had free access to a standard pellet diet and water. They were used in the experimental procedures after a minimum of three days of acclimatization to the housing conditions. Control and experimental groups consisted of 8–10 animals each. Treatment of the used laboratory animals in the present study was in full accordance with the respective Polish and European regulations and was approved by the Ethical Committee of the Jagiellonian University, Kraków, Poland (registration number 396/99 and ZI/UJ/219/2005).

Investigations of the skin-irritating activity of compounds KP-23, KP-23S and KP-23R in an ointment form were carried out on white guinea pigs. Experiments were carried out in groups of 9–10 animals. The shaven skin of the right or left side of the guinea-pig was exposed to 0.5 g of investigated compounds (applied but not rubbed in). The compound was reapplied everyday for 10 days. The exposed area was 5 × 5 cm. Results were assessed every day through a 10 day cycle. A positive reaction was defined as an erythematic reaction in 50% of the animals or the increase of thickness of the cutaneous folds. The thickness of the cutaneous fold was measured by micrometer (about the exactitude of indications of 0.05 mm) before the beginning of the experiment and on the last day of the experiment. The control group animals were given the basis (Lipobase) in such itself schema of application.

The influence on cellular hypersensitivity in guinea pigs was measured according to Maibach and Maguire [20], utilizing DNCB to sensitize the guinea pig skin. On the shaven and defatted skin of the right side of guinea-pigs (1.7 cm^2 surface), 1% acetone solution DNCB was applied for a total volume of

0.15 ml for eight days (the sensitizing phase). On the fifteenth day of the experiment, on the left side of guinea-pigs (surface 1.7 cm^2), a 0.1% DNCB solution was applied for a total volume of 0.015 ml (the expression phase). After the solvent vaporized, the area was dressed with sterilized gauze to avoid a secondary infection or contact with other allergens. The investigated compounds were applied individually as 0.5% ointment in expression phase and as 0.05% ointment in causing allergy phase. The appearance and the size of the erythema, the growth of the effusion and the swelling was observed after 24, 48, and 72 h from the fifteenth day of the experiment. The surface (S) of the erythema was measured planimetrically. A positive result was defined as the appearance of the erythema and an area of infiltration with a diameter greater than 5 mm 24–72 h after the last application. The local reaction in the form of the effusion and the swelling of the area where the antigen was applied was evaluated measuring the relative increase of the thickness of the cutaneous ($\Delta l/l_0$) fold by micrometer.

Determination of the mucous membranes-irritating influence of investigated compounds was measured on rabbit eyes, according to Norton et al. [24]. Tested compounds (0.1 ml of 1% solution) were applied to the rabbit's conjunctival sac. The conjunctival vascularization, width of the eyelid slit and the pupil were observed and recorded after 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, and 24 h after application. The skin fold and the eyelid growth were measured using micrometer.

PROCEDURES *IN VITRO*

Apparatus

The spectra and absorption assays were recorded using a Varian spectrophotometer model Cary 50 Bio. The measurements were performed at least in triplicate at room temperature. The antioxidant activity was assessed as described below.

Determination of antioxidant activity

The effects of diastereoisomers KP-23R and KP-23S on the hydroxyl radical formation in a Fenton-type reaction system has been investigated using 2-DR degradation assay, a simple and efficient method for the determination of rate constants for the reaction of hydroxyl radical with a wide range of biomolecules. In this method, added hydroxyl radical scavengers compete with 2-deoxyribose for the hydroxyl radicals produced and diminish chromogen formation [7, 10, 11].

The assay was performed as described by Halliwell et al. [10] with slight modification. A mixture of Fe(III)–EDTA + ascorbic acid H_2O_2 was selected as the Fenton medium for OH generation. The following reagents were added to clean glass tube in the following order: 0.95 ml 0.1 M phosphate buffer pH 7.4, 0.20 ml $FeCl_3$ 0.1 mM, 0.20 ml ascorbic acid 0.1 mM, 0.20 ml EDTA 1.04 mM, 0.20 ml H_2O_2 10 mM, and 0.25 ml 2-DR 20 mM. Bioactive compounds (KP-23S, KP-23R, lidocaine, tetracaine, or trolox) were added, before the iron salts, in various concentrations (1–15 mM). Reaction mixtures were incubated at 37°C for 1 h.

After incubation, the reaction was stopped by adding 2.0 ml of 2.8% (w/v) TCA, and the probe was mixed with 0.50 ml TBA 1% (w/v) in 0.5 M NaOH. The solutions were heated for 20 min at 100°C to develop colored malondialdehyde–thiobarbituric acid adduct (thiobarbituric acid reactive species, TBARS). The absorbance of a pink chromogen was measured at 532 nm against a blank (containing only buffer and DR). All of the analyses were performed in triplicate, and average values were taken.

The inhibition ratio (I) of DR degradation in percent by the examined compound was calculated according the following formula:

$$I(\%) = \left(\frac{A_0 - A}{A_0}\right) \cdot 100\%$$

where A_0 is the absorbance of the control reaction measured in the absence of hydroxyl radical scavenger and A is the absorbance of the test compound.

The rate determining step is the initial attack of OH radical with 2-DR. Competition kinetics involving a probe and a scavenger for $^{\bullet}OH$ reaction using various concentrations of the reactants should yield a straight line when 1/A is plotted as a function of scavenger concentration [S]. The rate of hydroxyl radical-scavenging reaction was calculated using the following formula:

$$\frac{1}{A} = \frac{1}{A_0}\left(1 + \frac{k_S[S]}{k_{DR}[DR]}\right)$$

where A is the absorbance in the presence of a scavenger S at concentration [S]; A_0 is the absorbance in the absence of a scavenger; k_S and k_{DR} are the rate constants of reactions of hydroxyl radicals with scavengers and with 2-DR, respectively; and [DR] is the concentration of 2-DR used in the experiment. We have used a typical value of 3.1×10^9 $M^{-1}s^{-1}$ for the second order rate constant of the competition reaction of a hydroxyl radical with 2-DR [10].

Statistical analysis

Results are expressed as the means ± SEM (*in vivo* studies) or the means ± standard deviation (*in vitro* assays). Statistical analysis was performed using the *post hoc* test after the one-way analysis of variance (ANOVA), or Student's *t*-test. The least significant difference (LSD) test (*in vitro* assays) or Dunnett's test (*in vivo* studies) were applied to determine differences between groups. Differences were considered significant when $p < 0.05$.

Results

IN VIVO EXPERIMENTS

Investigations of skin-irritating activity

The epicutaneous application of compounds KP-23S or KP-23R (0.5 g every 24 h through a 10 day cycle) did not induce perceptible changes in inflammation in the investigated animals. Neither an erythema nor swelling of the skin occurred after the application of the investigated compounds, either after the initial contact with the ointment or after the 10 day cycle of endermic exposure. Applied comparatively the basis as control, shows also no operation of originally irritating in applied system of experiences.

The influence on cellular hypersensitivity

Two fundamental symptoms of the late type hypersensitivity in guinea-pigs are erythema and the swelling of the skin. The greatest changes occurred 24–48 h after the application of the irritating dose. To estimate the relative affect of compounds KP-23S and KP-23R, the compounds were compared to the previously characterized compound DNCB. The compounds were compared based on their inductive capacity and the appearance of late type symptoms including erythema, vesiculation, infiltration and necrosis in the animal. Epidermic tests with compounds KP-23S or KP-23R (as 0.1% ointments) did not result in the appearance of an erythematic stain during the expression phase for any of the animals in the investigated group. The control animals also did not express symptoms. All animals tested with 0.1% DNCB showed positive symptoms 15 days after the application of the antigen. DNCB, the experimental contact-allergen, is a low-molecular chemical compound (with the hapten) that has allergenic properties. The cutaneous reaction after the renewed use of DNCB after the first initial contact with the skin can be a measure of the immunological sensitivity or, with the measure of the dynamics depending on the scale of the reaction, of late type hypersensitivity of an allergenic substance. Results of erythema after the use of the strongly antigenic DNCB and the comparison with investigated preparations KP-23S and KP-23R are represented in Table 1. Both in the experimental group (0.1% KP-23S, KP-23R or KP-23) and in the control group there was only a slight enlargement of the thickness of the cutaneous fold. The initial dose of 0.1% DNCB caused the increase in the thickness of the cutaneous fold from 36.9% (after 24 h) to 55.2% (after 72 h) when compared to the thickness of the fold before the application of the irritating dose (Tab. 1).

Determination of the mucous membranes-irritating influence

Compounds KP-23, KP-23S and KP-23R applied to the rabbit conjunctival sac in 1% solutions caused no perceivable changes. The palpebral gap stayed open during the entire observation. A slight redness of conjunctivae appeared directly after the application of the investigated compounds and disappeared after approximately 30 min. Compounds KP-23, KP-23S and KP-23R given in the investigated concentration (1%) did not cause the swelling of the palpebra or the epiphora and did not alter the thickness of the cutaneous fold of the palpebra. Changes were observed after 1% solution of the lidocaine and bupivacaine. Saline applied to the rabbit conjunctival sac under analogous conditions did not caused changes in the eye (Tab. 2).

Tab. 1. The influence of 2,4–dinitrochlorobenzene (DNCB) and investigated compounds on the formation of the effusion and the swelling in the reaction of the cellular hypersensitiveness. Δl – the increase of the thickness of the cutaneous fold, E(%) – the proportional growth of the thickness of the cutaneous fold. N = 12–14

	Δl (cm)														
	Control (basis)		Control (0.1% DNCB)		0.01% KP-23		0.01% KP-23R		0.01% KP-23S		0.01% LDC		0.01% BUP	
	X ± SEM	E %	X ± SEM	E%	X ± SEM	E%	X ± SEM	E%	X ± SEM	E%	X ± SEM	E%	X ± SEM	E%
0**	0.178 ± 0.008	0	0.156 [a] ± 0.008	0	0.166 [a] ± 0.006	0	0.186 [b] ± 0.01	0	0.160 [a] ± 0.006	0	0.167 [a] ± 0.003	0	0.184 [a] ± 0.007	0
24	0.181 ± 0.008	2.0	0.213 [a] ± 0.005	36.9	0.171 [a] ± 0.007	3.2	0.189 [b] ± 0.03	2.2	0.164 [a] ± 0.005	2.3	0.169 [a] ± 0.001	1.7	0.188 [a] ± 0.004	2.1
48	0.180 ± 0.001	1.4	0.195 [a] ± 0.005	25.0	0.169 [a] ± 0.006	1.6	0.189 [b] ± 0.01	1.9	0.163 [b] ± 0.087	1.8	0.169 [a] ± 0.005	1.2	0.187 [a] ± 0.007	1.9
72	0.180 ± 0.008	1.1	0.242 [b] ± 0.01	55.2	0.168 [a] ± 0.006	0.9	0.187 [b] ± 0.01	1.0	0.162 [a] ± 0.005	1.0	0.168 [a] ± 0.007	0.9	0.1854 [a] ± 0.013	0.8

Results are presented as the means ± SEM. t – the time of the observation (hours). N – the number of animals used in the experiment. 0** – the thickness of skin fold before application of dose, accepted for 100%. [a] $p < 0.001$; [b] $p < 0.01$

Tab. 2. The increase of thickness of skin fold (Δl) of the rabbit's eyelids after the application of investigated compounds to the conjunctival sac. (all compounds used as 1% concentrations)

Compounds	Eye-lid	Δ l						
		15 min	30 min	1 h	2 h	3 h	4 h	24 h
Control (0.9% NaCl)	UE	0.02	0.01	0.01	0.01	0	0	0
	BE	0.01	0	0.01	0	0	0	0
KP-23	UE	0	0.005	0	0.01	0.005	0	0
	BE	0	0	0	0.005	0	0	0
KP-23R	UE	0.02	0.015	0.015	0.01	0.005	0	0
	BE	0	0	0	0.005	0	0	0
KP-23S	UE	0.01	0	0.005	0.005	0	0	0
	BE	0.01	0	0.005	0	0	0	0
Bupivacaine	UE	0.01	0.005	0.005	0.005	0	0	0
	BE	0	0	0	0.005	0	0	0
Lidocaine	UE	0.01	0	0	0.01	0	0	0
	BE	0	0	0.01	0	0	0	0

Δl – the increase of the thickness of the cutaneous fold, expressed in cm. UE – the upper eyelid. BE – the bottom eyelid

The tested compounds caused no reactions, such as edema of the eyelid or lacrimation, and they did not influence the thickness of the skin fold. The skin of the guinea pig is a commonly used experimental model for estimating potential allergic properties of a compound. It is therefore safe to conclude that the hydroxyamine carane derivative and its R,S-diastereoisomers are safe for the skin. These tests provide compelling evidence that is necessary before conducting clinical trials.

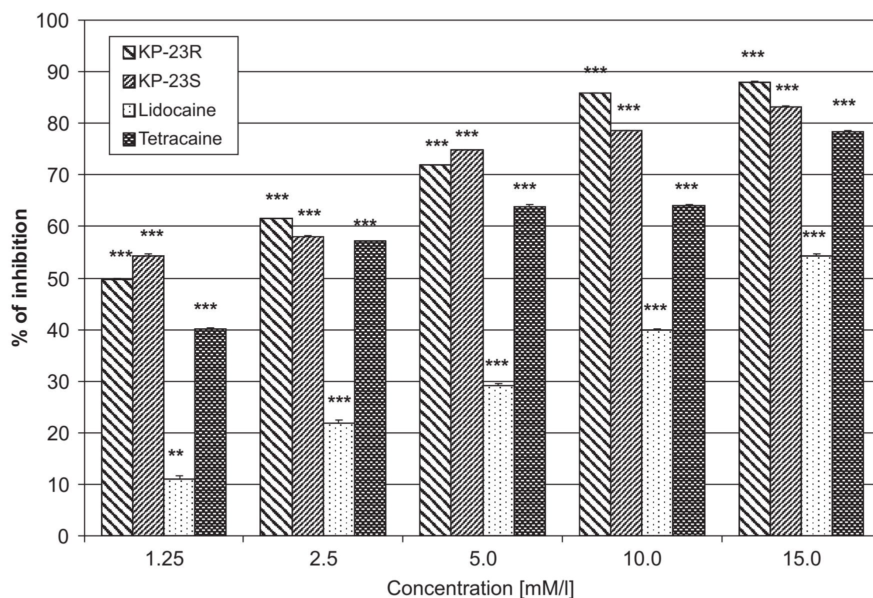

Fig. 2. Percentage of radical inhibition measured after 60 min of reaction with different concentrations of KP-23R, KP-23S, lidocaine and tetracaine. Values significantly different (** $p < 0.01$, *** $p < 0.001$; n = 3–6) *vs.* control

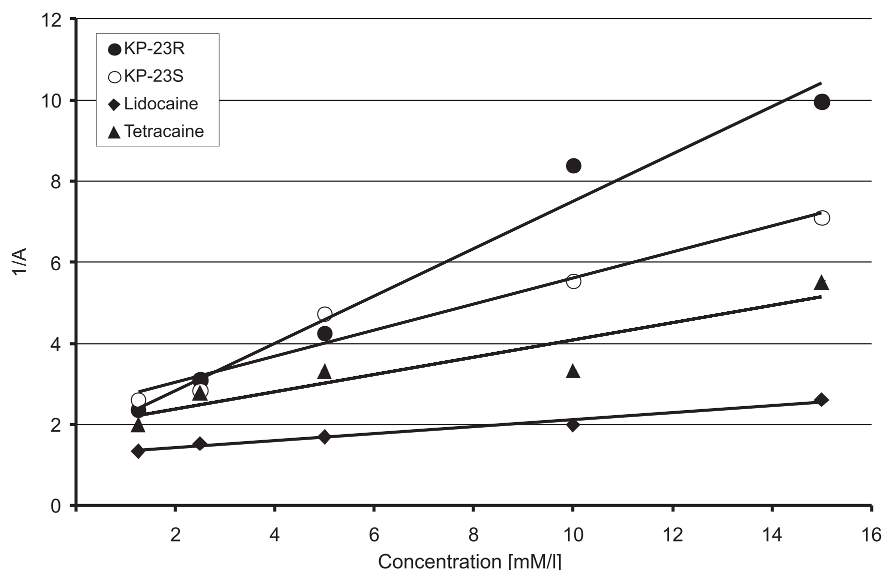

Fig. 3. Hydroxyl radical scavenging by varying concentrations of carane derivatives and local anesthetic activity drugs in the presence of 2-deoxyribose (20 mM) (the mean ± SD, RSD < 0.01). Reaction was carried out in phosphate buffer (pH 7.4) at 37°C for 60 min

IN VITRO EXPERIMENTS

Competition studies were performed to evaluate the effectiveness of the two potentials OH scavengers (KP-23R KP-23S) in protecting 20 mM of 2-DR from iron-mediated oxidative damage. Figure 2 shows that the percentage inhibitions of 2-DR degradation due to OH scavenging varied from 49.63% to 88% and from 52.0% to 83.12% for KP-23R and KP-23S, respectively, and were dose dependent. In this case, the largest inhibitory effect is caused by 15 mM KP-23R, followed by KP-23S, tetracaine and lidocaine.

Determination of the rate of hydroxyl radical scavenging reaction

The effect of the compounds on hydroxyl radical production was determined using 2-DR (20 mM) as the substrate. Figure 3 compares the rate constants of 2-DR degradation induced by the free radical-

Tab. 3. Second order rate constants (k_S) for reactions of reagents with hydroxyl radical determined by the competitive assay of the deoxyribose method

Compounds tested	Range [mM]	R^2	k_S [$M^{-1}s^{-1}$]
KP-23R	1.25–15.0	0.9889	3.55×10^{10}
KP-23S	1.25–15.0	0.9739	1.24×10^{10}
Lidocaine	1.25–15.0	0.9873	3.35×10^{9}
Tetracaine	1.25–15.0	0.9291	0.86×10^{9}

generating Fe(III)–EDTA, H_2O_2 and ascorbate system with increasing concentrations of KP-23R, KP-23S, tetracaine or lidocaine. The second-order rate constants of scavengers were proportional to the slopes of these lines, and the precision of the data was associated with the linear correlation coefficients. The calculated rate constants of hydroxyl radical-scavenging reactions are summarized in Table 3. It is known from the literature [30] that the second-order rate constant of model antioxidant trolox (water soluble tocopherol analogue) for OH reaction is 2.44×10^{12} $M^{-1}s^{-1}$. Under our reaction conditions, KP-23S and KP-23R inhibit the reaction with a second-order rate constant of 1.24×10^{10} $M^{-1}s^{-1}$ and 3.55×10^{10} $M^{-1}s^{-1}$, respectively. On the contrary, the second-order rate constant obtained for the known hydroxyl radical scavenger, lidocaine, was 3.35×10^{10} $M^{-1}s^{-1}$. This result was different to those obtained by Das (6×10^{10} $M^{-1}s^{-1}$) [4].

Discussion

The role of free radicals and active oxygen in the pathogenesis of human diseases including cancer, aging and atherosclerosis has been recognized. Interestingly, the antioxidant activity of antihistaminic agents, some local anesthetics and monoterpenes was also demonstrated [1, 5, 13, 25, 34]. Lidocaine was reported to protect erythrocytes from hemolysis induced by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). Lidocaine protected erythrocytes by scavenging radicals preferentially rather than by stabilizing membrane and has proprieties similar to an antiradical drug [31]. However, local anesthetics are known to induce apoptosis in clinically relevant concentrations [38]. Cano-Europa et al. [3] reported that lidocaine affects the oxidation and reduction environment and promotes increases of the oxidative markers both in the hippocampus and amygdala in different patterns.

In the deoxyribose method, radicals are generated in free solution by a mixture of Fe(III)–EDTA, H_2O_2 and ascorbate. It has been reported that KP-23R and KP-23S diastereoisomers are effective antioxidants in *in vitro* antioxidant assays, including assays quantifying the total antioxidant activity to scavenge the $ABTS^+$ radical cation [15]. These results do not exclude the possibility that KP-23R and KP-23S react directly with hydroxyl radical. The present study provides evidence that 1–15 mM KP-23R or KP-23S prevents 2-DR degradation by trapping $^{\bullet}OH$. This conclusion is based on the experiment in which hydroxyl radicals generated in a Fenton-type reaction attack and oxidized 20 mM of 2-DR. Oxidation products of 2-DR by hydroxyl radical, upon heating with thiobarbituric acid under acid conditions, yield chromogen with the maximum absorbance wavelength of 532 nm. If another compound is present in the solution, then it will compete with 2-DR and inhibit 2-DR degradation. This inhibition depends on the concentration of the compound in the reaction mixture and on its rate constant for the reaction with a hydroxyl radical [10].

The calculated second order rate constants for the reaction of KP-23R and KP-23S indicate that these compounds are good scavengers of hydroxyl radicals and are more effective that lidocaine and tetracaine. The second-order rate constant of trolox for $^{\bullet}OH$ reaction: $k = 2.44 \times 10^{12}$ $M^{-1}s^{-1}$ was found by Soobrattee et al. [30]. This study measured the hydroxyl radical scavenging rate constants of KP-23R and KP-23S as 1.24×10^{10} $M^{-1}s^{-1}$ and 3.55×10^{10} $M^{-1}s^{-1}$, respectively, suggesting that they are weaker scavenging activity than trolox.

An anti-inflammatory response to oxidative stress was also replicated in an *in vivo* model of glucose oxidase-induced paw edema in mice. In this acute inflammation animal model, the hydroxyl radicals induces tissue damage accompanied by the changes in the vascular permeability due to the enhanced production of various pro-inflammatory mediators including histamine, leukotrienes and prostaglandins [23, 27]. Moreover, the anti-inflammatory and antihistaminic actions of these compounds were also found in animal models where free radicals have been suggested to in-

duce inflammation. Free radicals and inflammation are linked; for example, during inflammation, neutrophils and macrophages excessively generate $O_2^{\bullet-}$ [35]. This precipitated the question of whether and in which degree enantiomers of the compound KP-23 can induce reactions cutaneous (toxic or allergenic). Cutaneous tests are universally used to determine the properties of allergen and irritating new substances. Experiments carried out using guinea-pigs showed that compounds KP-23S and KP-23R as well as the control, like KP-23 do not cause the inflammation of the skin. After both initial contact with the skin and repeated applications, 1.0% of either ointment caused no toxic cutaneous reactions in the animals studied. It is commonly accepted that the reactivity of human skin is similar to the reactivity of guinea pig skin, suggesting that these results can be generalized to human beings. Compounds KP-23, KP-23S and KP-23R did not produce late type allergic or toxic symptoms either in the location of the application of the drug or in another area as can sometime occur. However, DNCB caused a strong erythema, the swelling and the effusion characteristic of the contact-hypersensitivity in guinea pigs. In the present study, we observed similar results in the irritation of the mucous membranes of eyes of the rabbits treated with 1% (in 0.9% NaCl) solutions of both enantiomers of KP-23, lidocaine or bupivacaine. None of compounds caused visible changes in the appearance of the mucous membranes of the eye and did not cause the swelling of the palpebra or the epiphora. These results demonstrate that the two stereoisomers, KP-23S and KP-23R, and KP-23 do not irritate the skin and mucous membranes. This finding provides compelling evidence to begin clinical research on these compounds for use as local anesthetic.

This investigation showed that compound KP-23 and its R,S-diastereoisomers do not induce a late-type contact allergy. Neither erythema nor swelling was observed in the areas of the tested compounds application in the experimental group. It was also demonstrated that compound KP-23 and its R and S diastereoisomers evoke no visible reactions when applied to the conjunctival sac. The tested compounds caused no such reactions as edema of the eyelid, lacrimation, or an increase in the thickness of the skin fold. Because guinea pig skin is a widely accepted model for human skin, these compounds are most likely safe for use on human skin.

Conclusions

The present study shows that the new hydroxyamine carane derivatives, under the conditions of the experiments, did not irritate the skin or induce a late-type contact allergy in the guinea-pig. Additionally, we have demonstrated that KP-23S and KP-23R inhibit $^{\bullet}OH$ formation and, thus, 2-DR degradation induced by the ascorbate/Fe(III)/O_2 system. These monoterpene derivatives found to possess pronounced antioxidant effect may be at least in part related to its anti-inflammatory and antihistaminic activities.

However, because there are many contradictory reports concerning of oxidative activities of local anesthetics, one should carefully review studies of the antioxidative proprieties of new compounds. Further studies should consider the qualification abilities of *in vitro* scavenge on the other forms of reactive oxygen species, and the effect of high doses and their possible prooxidant effects.

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