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In vitro mutagenicity of valepotriates*

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Abstract. Valepotriates are epoxide-bearing triesters of the monoterpene alcohol 4,7-dimethylcyclopenta-(*c*)-pyrane isolated from the roots of several *Valerianacae* species. They are regarded as the main tranquilizing constituents of these drugs.

Although the valepotriates valtrate/isovaltrate (VAL) and dihydrovaltrate (DH-VAL) showed a strong alkylating activity against the nucleophilic agent 4-(*p*-nitrobenzyl)-pyridine (NBP), they were not clearly mutagenic for the strains TA98, TA100, TA1535, and TA1537 of *Salmonella typhimurium* or for the strains WP2 and WP2 uvrA$^-$ of *Escherichia coli* in the absence of a metabolic activation system (S9-mix). However, the valepotriates were mutagenic for TA100, WP2 and WP2 uvrA$^-$ at concentrations up to about 1.0 µmole/plate when S9-mix was added to the test system. With more than 1 µmole/plate the valepotriates were toxic in the presence of a metabolic activation system for all strains tested. The mutagenicity of the valepotriates was inversely related to the protein content of the S9-mix used. The mutagenicity and toxicity of the valepotriates could be inhibited when the S9-mix was preincubated with the esterase inhibitor paraoxon (1 mM) for 5 min before the test compounds and bacteria were added. Therefore, bioactivation of the valepotriates by an enzymatic hydrolysis of their ester groups is considered. This could be proven by activating the valepotriates with purified esterase.

Key words: Valepotriates – Mutagenicity – *Salmonella typhimurium* – *E. coli* – Microsome test – Paraoxon

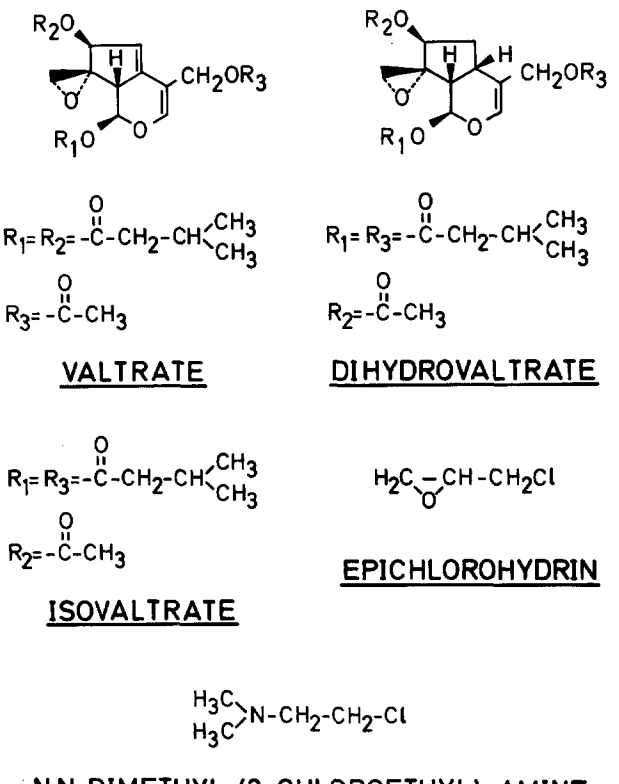

Fig. 1. Chemical structure of valepotriates, epichlorohydrin, and N,N-dimethyl-N-(2-chloroethyl)-amine

Introduction

The roots of several *Valerianaceae* contain epoxide-bearing valepotriates such as valtrate/isovaltrate (VAL) and dihydrovaltrate (DH-VAL) (Fig. 1) (Thies and Funke 1966; Thies 1966, 1967). Based on the evidence of animal experiments (Eickstedt and Rahman 1969; Mayer and Springer 1974; Holm et al. 1980) and clinical studies (Boeters 1968; Krüger 1969; Jansen 1977; Spassow and Gürowa-Postorino 1980), the valepotriates are regarded as the main effective sedative constituents of these roots.

Recently, it was shown that the valepotriates were cytotoxic to tumor cells, mouse early-hematopoietic progenitor cells, and human T-lymphycytes (Bountanh et al. 1981, 1983; Braun et al. 1982; Tortarolo et al. 1982). Possibly, the reason for this cytotoxicity may be the strong alkylating reactivity of the valepotriates due to their epoxide group as shown with 4-(*p*-nitrobenzyl)-pyridine (NBP) (Braun et al. 1982).

Stimulated by multiple reports on various directly alkylating agents showing a good correlation between the alkylating potency in the NBP test and the mutagenic potency in the Ames test (Hemminki and Falck 1979; Eder et al. 1982 a, b), we investigated the valepotriates for mutagenicity. The known direct mutagenic epichlorohydrin (EPI), and mus-

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tard-like N,N-dimethyl-N-(2-chloroethyl)-amine (DCEA) (Fig. 1) were used as reference substances. The latter cannot be affected by epoxide hydrolases, and was therefore included in this study.

Materials and methods

Test compounds. The isomer mixture valtrate/isovaltrate (VAL) was purified from highly enriched extracts of *Valeriana edulis*. A 2g sample of an enriched extract of *Valeriana edulis* (Fintzelberg, Andernach, FRG) was dissolved in 50 ml *n*-hexane. This solution was washed, dried over sodium sulfate, and evaporated to dryness (25–28° C) (Bountanh et al. 1981). The residue of about 0.5 g was redissolved in *n*-hexane and separated on a ethylene glycol desactivated aluminium oxide column (40 × 2 cm) using *n*-hexane as eluant. The fractions thus obtained were determined by UV spectroscopy at 254 nm (LKB, UvicordII). The fraction containing valtrate/isovaltrate was evaporated to dryness and purified using the same methods. HPLC analysis of the isomer mixture according to the method of Dossaji and Becker (1981) yielded a content of about 60% isovaltrate and 40% valtrate. Dihydrovaltrate (DH-VAL) was a gift from Kali-Chemie, Hannover, FRG. Epichlorohydrin (EPI) was purchased from Merck, Darmstadt, FRG, and paraoxon from Bayer, Leverkusen, FRG.

N,N-dimethyl-N-(2-chloroethyl)-amine (DCEA) was a gift from Hoechst, Frankfurt, FRG.

Esterase (carboxylic-ester hydrolase EC 3.1.1.1.) from porcine liver was from Boehringer, Mannheim, FRG.

Alkylating activity (NBP test). The incubation of the test compounds with NBP was performed as reported by Barbin et al. (1975): 2 ml 0.1 M tris-HCl buffer pH 6.6, 4 ml ethylenglycol, 135 mg 4-(*p*-nitrobenzyl)-pyridine (NBP), and 1 ml acetone, in which the test compound was dissolved, were mixed. After incubation for 90 min at 37° C, to 2 ml of the assay mixture, 1.67 ml triethylamine/acetone (1/1 v/v) were added. After 30 s the absorbance was measured at 560 nm (PM QIII, Zeiss). As blank, an assay free of the test compound was used.

Mutagenicity testing. All test compounds were tested for mutagenicity in *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, as well as in *Escherichia coli* WP2 and WP2 uvrA. The *S. typh.* strains were donated by B. N. Ames (Berkely, USA) and the *E. coli* strains by M. Green (Brighton, UK). The standard plate incorporation assay was performed according to the method of Ames et al. (1975) including the recommendations of Maron and Ames (1983). The same techniques and media were used for the *E. coli* reversion assay except that for this assay the top agar was supplemented with 0.25 µg/ml trytophan instead of histidine.

All substance concentrations were applied with 0.1 ml DMSO to the mutagenicity tests.

Metabolic activation systems. For metabolic activation, Arochlor 1254-induced rat liver homogenates were prepared according to the original protocol of Ames et al. (1975) from male Wistar rats (180 g). The S9-mix generally contained per milliliter: 8 µmoles $MgCl_2$, 33 µmoles KCl, 5 moles glucose-6-phosphate, 100 µmoles sodium-phosphate pH 7.4 and 1.2 mg S9-protein. 0.5 ml S9-mix was subjected per plate.

In order to study the effect of paraoxon, the S9-mix was preincubated without and with paraoxon (1 mM) for 5 min at 37° C and then plated with the test substance, bacteria, and topagar. For preparation of cytosol and microsomes, the thawed S9-fraction was centrifuged for 1 h with 100,000 *g*. The supernatant cytosolic fraction contained two-thirds of the original protein amount and the pelleted microsomes contained one-third. Therefore, 0.4 mg cytosol and 0.2 mg microsomal protein were used per plate in the mutagenicity tests. The microsomal activation system contained the same compounds as the S9-mix and was supplemented with 2 U glucose-6-phosphate dehydrogenase per plate. Mutagenicity tests with esterase were carried out in the same way as with S9-mix, using 0.5 U esterase dissolved in 0.5 ml sodium phosphate (0.2M, pH 7.4) instead of S9-mix.

Results

As shown in Fig. 2, valtrate (VAL) alkylates NBP more strongly than the mustard-like N,N-dimethyl-N-(2-chloroethyl) amine (DCEA) or epichlorohydrin (EPI), while the alkylating potency of dihydrovaltrate (DH-VAL) is considerably weaker.

However, in the bacterial mutagenicity assay without activation, EPI and DCEA showed a distinct, dose-dependent, direct mutagenic activity with TA100, TA1535, WP2, and WP2 uvrA$^-$, while VAL and DH-VAL were inactive to all strains tested (Table 1). Surprisingly, in the presence of S9-mix, VAL, and DH-VAL had substantial activity, with TA100, WP2, and WP2 uvrA$^-$ in the range between 0.1 and 1.0 µmole/plate. At higher concentrations, the valepotriates were toxic to all six strains. In the presence of S9-mix, the revertant numbers increased with VAL and DH-VAL in a dose-dependent manner. The number was twice that of the spontaneous revertants (Table 1). In contrast to the results obtained with the valepotriates, with EPI and DCEA the mutagenicity was partially decreased in the presence of the S9-mix. The mutagenicity of the valepotriates was inversely related to the protein content of the S9-mix used (Table 2).

As shown in Fig. 3, the metabolic activation of the valepotriates resulted more from microsomal enzymes than from enzymes of the cytosol. The mutagenicity and toxicity could be

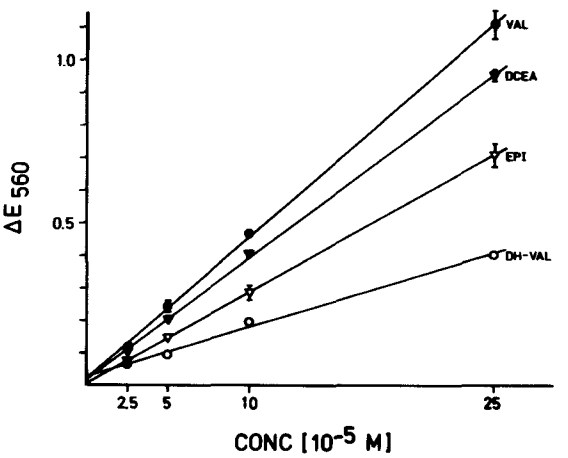

Fig. 2. Dose-dependent alkylation of NBP by valtrate/isovaltrate (*VAL*, MW 422.5), N,N-dimethyl-N-(2-chloroethyl)-amine (*DCEA*, MW 107.7), epichlorohydrin (*EPI*, MW 92.5) and dihydrovaltrate (*DH-VAL*, MW 424.5); incubation time 90 min; medium pH 6.6

Table 1. Mutagenic activity of valtrate, dihydrovaltrate, epichlorohydrin, and N,N-dimethyl-2-chloroethylamine

Compound	Conc. µmole	Revertants per plate											
	plate	TA98		TA100		TA1535		TA1537		WP2		WP2	uvrA⁻
		−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
DMSO		33	37	120	74	28	18	8	10	41	52	30	60
VAL	0.1	33	44	104	91	47	13	6	6	50	90	76	110
	0.33	36	44	140	97	23	18	11	8	52	87	75	96
	1.0	38	34	129	158	33	13	10	+	60	64	68	84
	3.3	49	+	148	126	28	+	7	+	66	33	81	66
	10.0	51	+	147	34	37	+	13	+	74	43	77	45
DH-VAL	0.1	31	42	140	86	22	24	8	8	50	77	68	117
	0.33	34	38	148	171	29	8	9	3	47	114	51	141
	1.0	37	23	167	112	27	+	7	+	46	+	61	+
	3.33	31	+	156	+	31	+	8	+	53	+	64	+
	10.0	31	+	152	+	42	+	9	+	52	+	79	+
EPI	0.1	32	37	138	67	74	24	8	7	60	72	64	81
	0.33	38	28	172	75	142	44	8	5	55	74	80	83
	1.0	33	33	305	90	286	94	10	5	107	106	114	123
	0.33	33	33	596	165	590	266	10	6	175	191	231	201
	10.0	48	37	1,177	604	850	633	6	3	406	420	444	475
DCEA	0.1	33	35	144	71	68	62	10	11	971	329	684	320
	0.33	25	32	165	96	132	132	9	10	909	626	1,008	517
	1.0	32	25	268	135	267	294	12	7	1,238	894	1,210	680
	0.33	34	40	487	231	597	581	9	10	1,316	1,141	1,360	920
	10.0	39	35	822	218	913	933	10	10	1,410	1,170	1,276	771
2-AA	2 µg		1,641		979		206		50		150		220
2-NF	10 µg	986											
NaN_3	4 µg			1,110		1,130							
MMS	1 µg									1,071		771	
9-AAc	10 µg							231					

2-AA: 2-aminoanthracene; 2-NF: 2-nitrofluorene; NaN_3: sodium azide; MMS: methyl methanesulfonate; 9-AAc: 9-aminoacridine; revertants = mean of six plates; + toxic for bacteria

Table 2. Metabolic activation of valtrate, dihydrovaltrate, and 2-aminoanthracene as related to the amount of S9-protein

Strain	S9-protein	Revertants per plate			
	plate (mg)	DMSO	VAL[a]	DH-VAL[b]	2-AA[c]
TA100	0.6	121	260	224	1,870
	1.2	136	237	205	1,720
	2.4	133	227	165	1,001
TA98	0.6	34	56	37	2,080
	1.2	42	49	51	1,711
	2.4	42	50	39	1,105
WP2 uvrA⁻	0.6	59	89	177	157
	1.2	58	77	169	105
	2.4	58	77	101	84
WP2	0.6	65	94	150	163
	1.2	57	85	129	98
	2.4	64	82	96	83

[a] 1 µmole/plate
[b] 0.33 µmol/plate
[c] 2 µg/plate

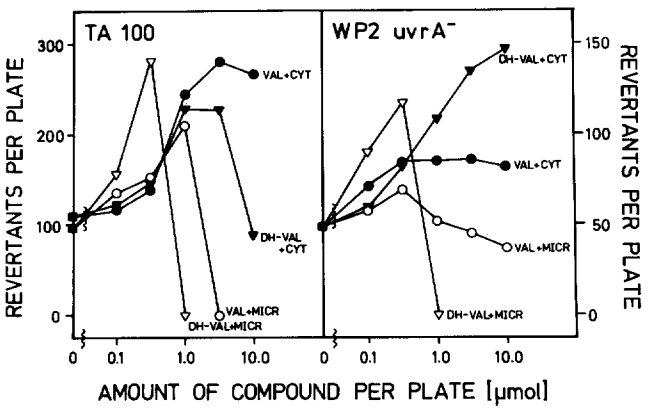

Fig. 3. Response of *S. typh.* TA100 and *E. coli* WP2 uvrA⁻ to valepotriates in the presence of a microsomal (*MICR*) or cytosolic (*CYT*) fraction. As positive control for metabolic activity of the microsomal fraction 1 µg benzo(a)pyrene gave 580 revertants

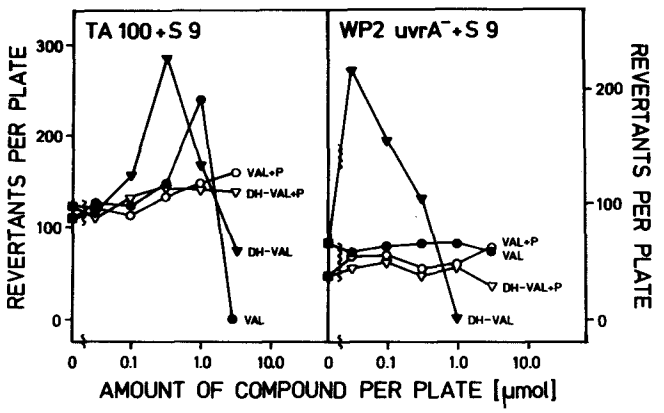

Fig. 4. Response of *S. typh.* TA100 and *E. coli* WP2 uvrA⁻ to valepotriates in the presence of an S9-mix without and with preincubation with paraoxon (*P*).

Table 3. Mutagenicity of valtrate and dihydrovaltrate after activation with 0.5 U purified esterase

μmole	TA 100 revertants per plate	
plate	VAL	DH-VAL
DMSO	96	96
0.01	–	115
0.03	–	154
0.10	145	208
0.20	–	275
0.33	154	207
1.00	240	+
2.00	289	+
3.33	+	+

The number of revertants is the mean of two independent tests each in triplicate; + toxic, clear background; – not tested

observed at lower concentrations of the test compounds in the presence of the microsomal fraction of the S9-fraction than in the presence of the cytosol fraction.

The metabolic activation by the S9-mix was inhibited completely when the activation system was preincubated with paraoxon (Fig. 4). The mutagenic and toxic potency of VAL and DH-VAL was abolished under these conditions.

Tests of VAL and DH-VAL with 0.5 U purified esterase from porcine liver instead of S9-mix showed a dose-dependent mutagenicity and toxicity with strain TA100 at similar dose ranges as with S9-mix (Table 3).

Discussion

The results obtained are surprising. Although the valepotriates have to be regarded as strong direct alkylating agents due to their reactivity in the NBP test, they showed no direct mutagenicity in the Ames test up to 10.0 μmole/plate. Recently, Glatt et al. (1983) also found DH-VAL and VAL to be nonmutagenic, but they could test these drugs only up to 0.24 and 0.7 μmole/plate, respectively. At higher concentrations, they observed a macroscopically visible precipitation of the test compound on the plate. We used 0.1 ml DMSO as solving agent instead of 0.05 ml (Glatt et al. 1983) and did not observe such precipitations in our experiments. Thus, the valepotriates do not show the good correlation between the direct alkylating potency in the NBP test and the activity in the Ames test which was observed with DCEA, EPI, and other epoxides (Hemminki and Falck 1979).

On the other hand, the valepotriates developed a slight, but clear, mutagenicity and cytotoxicity in the presence of S9-mix for the strains TA100, WP2, and WP2 uvrA⁻; under the same conditions the mutagenicity of DCEA and EPI was decreased. The inactivation of direct mutagenic compounds, especially epoxides, by a metabolic activation system was previously reported (Voogd et al. 1981; Yoshikawa et al. 1980; El-Tantawy and Hammock 1980; Oesch and Glatt 1976; Wood et al. 1976). Glatt et al. (1983) did not find mutagenic properties of the valepotriates in the presence of S9-mix up to their highest test concentration. We could demonstrate the mutagenic response for VAL and DH-VAL up to 1 μmole/plate and 0.33 μmole/plate with several tests (Tables 1 and 2; Figs. 3 and 4). Furthermore, a differing S9-protein content in the test systems could also be responsible, since a modulating effect of the protein amount in the assay could be shown.

The detoxification of epoxides is caused by enzymes of the 100,000 *g* supernatant fraction, especially by glutathione S-epoxide transferases (Yoshikawa et al. 1980; El-Tantawy and Hemmock 1980) as well as by the microsomal epoxide hydratase (Yoshikawa et al. 1980; El-Tantawy and Hammock 1980; Rossi et al. 1983). The valepotriates, in contrast, are activated by both the microsomal system and the cytosolic fraction, and therefore the epoxide group cannot be regarded per se as the toxic moiety of the valepotriate structure. Due to the inhibition of valepotriate activation by paraoxon, carboxylesterases and deacetylases which in rat hepatocytes are located predominantly in the endoplasmatic reticulum, have to be considered as responsible for bioactivation (Bos et al. 1981; Kaneda et al. 1981; Fu et al. 1982; Auckermann et al. 1983; Staiano et al. 1983). This suggestion is supported by the results obtained with purified esterase.

Although the valepotriates are activated by esterase, the structures of the mutagenic metabolites remain unclear. On the one hand steric hindrance of the reaction of the epoxide group with macromolecular targets might be diminished after hydrolysis of R_1 and/or R_2 by esterases. On the other hand, the decomposition of the resulting highly unstable alcohols forming reactive α,β-unsaturated aldehydes (Thiess 1969) usually results in the loss of the epoxide group. Numerous compounds exhibiting this structure have been known to be genotoxic and cytotoxic (Shauenstein et al. 1977; Esterbauer et al. 1976; Lutz et al. 1982). Therefore, metabolites without an epoxide group formed by valepotriate hydrolysis may also be responsible for the observed biological effects.

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