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A NOVEL BIOLOGICALLY ACTIVE SELENO-ORGANIC COMPOUND—VI

PROTECTION BY EBSELEN (PZ 51) AGAINST GALACTOSAMINE/ENDOTOXIN-INDUCED HEPATITIS IN MICE

ALBRECHT WENDEL and GISA TIEGS

Physiologisch-Chemisches Institut der Universitat, Hoppe-Seyler-Str 1, D-7400 Tubingen, Federal Republic of Germany

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Abstract—Male albino NMRI mice were given 700 mg/kg galactosamine and 33 μg/kg salmonella endotoxin intraperitoneally After 9 hr, serum sorbitol dehydrogenase activity had risen from 60 to 7320 U/l, SGOT from 90 to 5580, and SGPT from 70 to 10,440 When a similar dose of galactosamine alone or endotoxin alone was given, no significant liver injury was found. Animals pre-treated with an oral dose of ebselen (600 mg/kg 1–3 hr before galactosamine/endotoxin administration) were fully protected against this type of hepatitis When pretreated 1 hr before intoxication with different doses of ebselen, significant dose-dependent reduction of serum enzyme activities was observed at doses higher than 1 mg/kg. After pre-treatment with 6 mg/kg ebselen, no biochemical or histological signs of liver lesions were detectable 36 hr after intoxication In order to comparatively evaluate the model used, several established anti-inflammatory drugs were administered at doses which showed 50% effectiveness in preventing carageenan paw edema A dose of 200 μg/kg dexamethasone, or 9 mg/kg indomethacin abolished galactosamine/endotoxin-induced enzyme release in our animals, as did the lipoxygenase pathway inhibitor diethylcarbamazine (78 mg/kg) In contrast, administration of cyclooxygenase pathway inhibitors such as aspirin (220 mg/kg) or ibuprofen (45 mg/kg) failed to prevent hepatitis The effect of ebselen was also investigated in four different models of acute drug-induced liver damage A dose of 600 mg/kg of the organic selenium compound was ineffective or weakly active in benzo(α)pyrene- or phenobarbital-treated mice which were intoxicated by intraperitoneal administration of 350 or 400 mg/kg body weight of paracetamol Similarly negative results were obtained against bromobenzene-induced hepatotoxicity (520 mg/kg bromobenzene i p), carbon tetrachloride intoxication (3 2 g/kg), or allyl alcohol-induced liver damage (60 mg/kg) The selective efficacy of ebselen against galactosamine/endotoxin induced liver damage is interpreted in terms of its recently recognized ability to inhibit the formation of leukotrienes

Because of its glutathione peroxidase-like activity [1, 2] the anti-inflammatory compound ebselen (PZ 51, 2-phenyl-1,2-benzoisoselenazol-3(2H)-one) has attracted interest as an anti-oxidant in cellular [3, 4] as well as subcellular [1] systems. In the preceding paper of this series [5] we showed that ebselen inhibited leukotriene B_4 (LTB_4) production from endogenous arachidonic acid by rat polymorphonuclear leukocytes at a half-maximal inhibitory concentration of 20 μmoles/l Unlike purified glutathione peroxidase in the presence of its substrate GSH, the inhibition due to ebselen was shown not to be caused by enhanced reduction of 5-HPETE (5-hydroperoxy-eicosatetraenoic acid) to 5-HETE (5-hydroxyeicosatetraenoic acid) Rather ebselen seems to act directly on leukocyte lipoxygenase At lower concentrations, ebselen also isomerizes LTB_4 to an inactive *trans* isomer *in vitro* [6]

A recent report demonstrated that *in-vivo* administration of endotoxin to rats led to an immediate increase in leukotriene secretion into the bile [7] It is also known that galactosamine-induced hepatitis in mice, rats and rabbits is potentiated by simultaneous administration of endotoxin and shows large inter- and intra-species variations with respect to the susceptibility of the animals. Since a protective effect of inhibitors of leukotriene synthesis or action has been described against GalN/endotoxin-induced lethality in mice [8], we were interested in investigating the *in-vivo* effect of ebselen in a similar model

MATERIALS AND METHODS

Male albino mice (strain NMRI, Han) were purchased from the Zentralinstitut fur Versuchstiere, Hannover, F.R G They were kept at least one week on the standard diet C 1000 (Altromin, Lage) with free access to food and water under environmentally controlled conditions Their average weight was 35 g. Monooxygenase activity was induced by giving 1 mg/ml phenobarbital in the drinking water for 5 days. Phenobarbital was withdrawn the last day before the experiments Induction by benzo(α)-pyrene was carried out by injection of 20 mg/kg benzo(α)pyrene on three consecutive days. In order to fix the diurnally varying hepatic glutathione [9] these animals were fed a liquid sucrose diet for the 48 hr prior to the paracetamol experiments

* Abbreviations SGOT, serum aspartate aminotransferase (EC 2 6 1 2), SGPT, serum alanine aminotransferase (EC 2 6 1 1), SDH, sorbitol dehydrogenase (EC 1.1 1 14), GalN, galactosamine

Blood was withdrawn by heart puncture into 2 5% heparin The livers were perfused for 1 min with ice cold 0 9% NaCl via the portal vein, then removed and immediately frozen in liquid nitrogen. They were stored for no longer than 2 days. They were homogenized in 3% metaphosphoric acid containing 1 mM EDTA prior to enzymatic assay of glutathione content

Ebselen and analogues were gifts from Nattermann & Cie (GmbH, Cologne, F.R G) Galactosamine HCl was purchased from Roth Chemicals (Karlsruhe) and endotoxin (*Salmonella abortus equi* lipopolysaccharide) from Sigma (St Louis, MO). Paracetamol and diethylcarbamazine were from Sigma, bromobenzene, CCl_4 and dexamethasone from Merck (Darmstadt), aspirin from Bayer (Leverkusen, F.R.G) and ibuprofen from Klinge (Munchen, F R G).

Serum enzyme activities were determined according to Bergmeyer [10]. Total soluble non-protein-bound glutathione was measured according to Tietze's method [11]. Protein was assayed by Lowry's procedure using bovine serum albumin (Sigma) as standard All data are given as means ± standard deviation (S D.) Statistical significance was evaluated according to Student's *t*-test.

For histopathological evaluation, the livers of the animals were perfused after the experiment with 10% phosphate-buffered formalin and immediately fixed in this medium. Sections were stained with eosine/hematoxiline and graded by Dr. Heide Schmid, Pathology Department, without knowledge of their origin

RESULTS

When male mice were given a single injection of 33 μg/kg (or up to 15 mg/kg) endotoxin, no significant increase in serum transaminase levels was observed within the next 9 hr Similar observations were made when a dose of 700 mg/kg galactosamine was administered as a single injection However, the combination of these two toxins led to very high increases in enzyme release into the circulation in most of the animals (Table 1, basal values vs control) No enhancement of serum γ-glutamyl transpeptidase activity over the basal levels of 1 3 ± 0 5 mU/l was observed We noticed that roughly ten per cent of our animals, however, did not respond at all to this combined GalN/endotoxin treatment Therefore, these animals are listed separately First of all, groups of eight animals were pre-treated 3, 2 or 1 hr before GalN/endotoxin with a maximum oral dose of 600 mg/kg ebselen Independent of the pre-treatment regimen, all animals were optimally protected as assessed by enzyme release. Subsequently, the dose-dependence of the effect of ebselen given 1 hr before GalN/endotoxin was investigated The results in Table 1 demonstrate a significant dose-dependent protection of ebselen against GalN/endotoxin intoxication at oral doses higher than 1 mg/kg within the time of the survival of the control animals, i e up to 9 hr. Data in Table 1 demonstrate moreover the selective efficacy of ebselen *in vivo* compared to analogues which turned out to have no glutathione peroxidase-like activity *in vitro* [2]

In order to discriminate between a protection and a delaying effect of ebselen on the development of the injury GalN/endotoxin-intoxicated mice were pretreated with 600, 60 or 6 mg/kg ebselen 1 hr before intoxication and observed until 36 hr later After this time, no significant enhancement of serum enzyme activities compared to untreated controls was found at any dose of ebselen applied The following specific activities were measured, e g in a group of seven animals treated with the lowest dose of ebselen, i.e 6 mg/kg. SDH = 150 ± 80, SGOT = 70 ± 50, SGPT = 90 ± 50 (control data in Table 1) Essentially no histopathological lesions were detectable in the liver sections from these animals This experiment suggests that a real protection rather than a delay of GalN/endotoxin-induced hepatotoxicity had taken place

In order to evaluate the nature of the model at the doses of GalN and endotoxin used here, we studied the effect of some agents which interfere with arachidonate metabolism For these experiments we chose doses that correspond to the ED_{50} of these compounds in rat carageenan induced paw edema [12] The results in Table 2 indicate that compounds which preferentially block the cyclooxygenase pathway

Table 1 Dose-dependent protection by oral ebselen pre-treatment against galactosamine/endotoxin-induced hepatitis in mice and specificity of the compound compared to some analogues

Additional treatment	SDH	SGOT	SGPT	R	NR	M
Basal (without treatment)	60 ± 40	90 ± 10	70 ± 30	(10)		
Tylose (control)	7320 ± 5630	5580 ± 5120	10,440 ± 8640	49	5	9/54
600 mg/kg Ebselen	100 ± 66***	120 ± 50***	140 ± 100***	12	1	0/13
60 mg/kg Ebselen	560 ± 640***	340 ± 350***	730 ± 890***	21	3	2/24
6 mg/kg Ebselen	880 ± 1450***	770 ± 1150***	1300 ± 2220***	21	3	1/24
1 mg/kg Ebselen	3870 ± 4190$^{n.s}$	3430 ± 4120$^{n.s}$	5650 ± 6130$^{n.s}$	16	0	3/16
0 6 mg/kg Ebselen	4540 ± 3800$^{n.s}$	4620 ± 3920$^{n.s}$	6360 ± 5130$^{n.s}$	3	0	0/3
0 1 mg/kg Ebselen	5490 ± 4690$^{n.s}$	3890 ± 3680$^{n.s}$	7900 ± 7330$^{n.s}$	8	0	4/8
510 mg/kg sulfuranalogue PZ 25†	3020 ± 3320$^{n.s}$	2140 ± 1580$^{n.s}$	4100 ± 3860$^{n.s}$	8	0	1/8
635 mg/kg Se-methyl derivative‡	4630 ± 3730$^{n.s}$	3130 ± 2550$^{n.s}$	5990 ± 4820$^{n.s}$	8	0	4/8

† 2-Phenyl-1,2-benzisothiazol-3(2H)-on, ‡ 2-Methylselenobenzanilide

Galactosamine (700 mg/mg) and endotoxin (33 μg/kg) were injected intraperitoneally 1 hr later as a mixture in phosphate-buffered saline Serum enzyme activities were assayed after nine hours and are given in U/l R responders, NR, non-responders, M, mortality within 9 hr , ***, $P \leq 0.001$ of pre-treated against control animals

Table 2 Influence of various anti-inflammatory agents on galactosamine/endotoxin hepatitis in mice

Additional treatment	SDH	SGOT	SGPT	R	NR	M
Tylose (control)	7320 ± 5630	5580 ± 5120	10,440 ± 8640	49	5	9/54
220 mg/kg Aspirin	5930 ± 9940 n s	3370 ± 4080 n s	8010 ± 13,230 n s	8	0	1/8
45 mg/kg Ibuprofen	5460 ± 6600 n s	2840 ± 2800 n s	7160 ± 7850 n s	10	0	0/10
9 mg/kg Indomethacin	400 ± 460**	480 ± 475**	730 ± 1040**	8	—	0/8
78 mg/kg Diethylcarbamazine	190 ± 115***	270 ± 90***	290 ± 180***	10	—	0/10
200 μg/kg Dexamethasone	110 ± 60***	140 ± 100**	100 ± 60***	8	—	0/8

Dexamethasone was injected intraperitoneally 1 hr before GalN/endotoxin in phosphate-buffered saline Diethylcarbamazine was injected intraperitoneally every 45 min between 0 and 6 hr ** = $P \leq 0.01$, *** = $P \leq 0.001$ of treated vs control animals

such as aspirin or ibuprofen had no significant effect on GalN/endotoxin-induced hepatitis Indomethacin significantly reduced serum enzyme activity release in this model at a dose where the drug is able to inhibit cyclooxygenase as well as phospholipase A_2 [14]. Likewise, dexamethasone which indirectly inhibits phospholipase A_2 also prevented the signs of fulminant GalN/endotoxin-induced hepatitis. Similar results were obtained when mice were treated with repeated injections of the LTA_4 synthesis inhibitor diethyl carbamazine which has a very short half-life *in vivo* [13] These experiments suggest that the model used here is sensitive to modulators of eicosanoid metabolism which prevent the formation of leukotrienes. These findings confirm and extend earlier observations by others [8, 13, 14].

Finally, we compared the efficacy of ebselen against experimental liver injury in different models. Table 3 contains the results of some experiments where liver lesions in mice were produced by compounds which are metabolized to form reactive intermediates One example for cytosolic production of a reactive intermediate is allyl alcohol, the others represent common hepatotoxins handled by the microsomal monooxygenase It has previously been shown that inhibition of the metabolism of paracetamol on the one hand [15, 16] or a fortification of the glutathione redox system on the other hand [17, 18] prevented this type of acute liver damage. The data in Table 3 show that ebselen pre-treatment resulted in no or barely significant protection against paracetamol-induced liver necrosis Essentially similar results with no significant protection by ebselen were obtained in the model of bromobenzene-, or carbon tetrachloride-induced liver damage. Allyl alcohol intoxication, in contrast, leads to liver injury by consecutive reactions of the primary toxic metabolite acrolein which is formed via the cytosolic alcohol dehydrogenase reaction [19]. As expected, ebselen pre-treatment of mice before administration of allyl alcohol failed to show any protective effect These findings do, however, not exclude beneficial effects of ebselen against drug-induced liver lesions under less drastic conditions, e.g in chronic disease states. These acute models are not suitable to study pharmacological actions of this kind

The experiments in Table 3 show also that ebselen itself does not interfere with the utilization of hepatic GSH on the one hand, nor does it deplete hepatic GSH via oxidation to GSSG, as would have been presumed if ebselen were acting like glutathione peroxidase A similar GSH depletion *in vivo* after paracetamol or bromobenzene administration in the presence or absence of ebselen indicated that the metabolism of the xenobiotics was not impaired in the ebselen-pretreated animals.

Table 3 Effect of oral ebselen pre-treatment against chemically induced acute liver injuries in mice

Treatment	SDH	SGOT	SGPT	Hepatic GSH + 2 GSSG (nmol/mg prot)	n =	M
Vehicle (control)†	110 ± 80	195 ± 50	250 ± 105	26 ± 2	7	0
350 mg/kg paracetamol†	860 ± 730	1000 ± 1170	1430 ± 1210	12 ± 3 7	13	0
Paracetamol + ebselen†	1270 ± 1260	1350 ± 1090	2150 ± 1930	8 ± 6	13	0
Ebselen†	100 ± 60	375 ± 250	350 ± 140	25 ± 2	6	0
400 mg/kg paracetamol‡	1530 ± 1260	900 ± 800	1150 ± 1040	1 7 ± 1	6	0
Paracetamol + ebselen‡	390 ± 240*	570 ± 320	360 ± 130*	2 9 ± 0 9	6	0
520 mg/kg bromobenzene†	2060 ± 1250	1160 ± 750	2060 ± 1250	1 2 ± 0 5	6	3
Bromobenzene + ebselen†	1650 ± 1110	1460 ± 820	1910 ± 1200	2 ± 0 4	6	3
3 2 g/kg CCl_4	500 ± 200	2790 ± 780	890 ± 380	23 ± 6	8	1
CCl_4 + ebselen	415 ± 140	2070 ± 640	780 ± 320	22 ± 2	8	0
60 mg/kg allylalcohol	1070 ± 625	2150 ± 2390	1800 ± 2420	6 0 ± 4 7	8	3
Allylalcohol + ebselen	3200 ± 2990	2940 ± 2690	4630 ± 7240	2 0 ± 2 8	8	8

Paracetamol was given in DMSO/0 9% NaCl, 40 60 (v/v), bromobenzene as a 5% solution in vegetable oil, CCl_4 as a 50% solution in vegetable oil, allylalcohol as a 2% solution in 0 9% NaCl Serum enzymes and liver glutathione were measured four hours after dosage of the hepatotoxins paracetamol, CCl_4, or allylalcohol and 7 hr after bromobenzene † Phenobarbital-pretreated animals, ‡ benzo(α)pyrene-pretreated animals, * $P \leq 0.05$% of intoxicated vs ebselen-treated mice

DISCUSSION

Much of the pathophysiology in endotoxin shock is closely related to reactions exerted by lipid mediators of anaphylaxis and inflammatory responses, including the leukotrienes The formation of the chemotactic agent LTB_4 is inhibited in rat neutrophils in the presence of ebselen in a dose-related and specific way [5, 6] On the other hand, an increased concentration of leukotrienes in the bile within the first two hours following endotoxin treatment was observed as an early reversible event in rats *in vivo* [7] It was therefore obvious to investigate the preventative effect of ebselen in an animal model closely related to human viral hepatitis under conditions whereby mice had been sensitized to GalN-induced liver lesions by endotoxin [13, 20]

Our results show a protective effect of ebselen against GalN/endotoxin hepatitis with a similar dose-dependence as found for the anti-inflammatory activity of the compound against cobra-venom factor-induced edema (IC_{50} = 56 mg/kg p.o) [21] Bearing in mind that conventional non-steroidal anti-inflammatory drugs had only moderate activity in this inflammatory model, these findings suggest that the common efficacy of ebselen in the hepatitis model and against inflammation might be related to its ability to inhibit the lipoxygenase pathway In line with this interpretation, compounds decreasing either the availability of arachidonic acid or its metabolism to leukotrienes (Table 2) resulted in an apparent protection against GalN/endotoxin hepatitis, while mere inhibitors of the prostaglandin pathway had no significant effect

Regarding the latter statement, it should be pointed out that indomethacin, in addition to its well-known inhibition of cyclo-oxygenase, also inhibits phospholipase A_2 [14] and prevented endotoxin-induced phospholipase activation in rats [22]

In order to gain more mechanistic insight into the hepatoprotective effect of ebselen, we extended our *in-vivo* studies to several different models of chemically induced liver injury, in which alkylation or peroxidation mechanisms are involved in the primary lesion mechanism (for a review cf ref 23) Since ebselen also exhibits antioxidant properties under certain conditions *in vitro* [4] we would have anticipated that this property or the glutathione peroxidase-like activity of the compound [1, 2] would result in a protective effect against reactive metabolite liver injury models as well The results in Table 3 make this interpretation unlikely They also show that high doses of ebselen alone do not deplete hepatic glutathione and do not impair the utilization of glutathione for the phase II metabolism of paracetamol or bromobenzene Likewise, the cytosolic metabolism of allyl alcohol as well as its deleterious action on the liver is not affected by ebselen pretreatment of the animals We interpret these experiments in the sense that ebselen is not likely to interact as such with reactive intermediates including activated oxygen species stemming from microsomal monooxygenase metabolism, or in the case of allyl alcohol, with cytosolically formed acrolein

These findings suggest that rather than a general antioxidant property, inhibition of eicosanoid formation and/or release may underly the hepatoprotective as well as the anti-inflammatory activity of ebselen. A promising prospect for the pharmacological application of ebselen might be its therapeutic use against inflammatory liver disease, or, in general against inflammatory conditions associated with cellular infiltration

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