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Streptococcal Cell Wall-induced Systemic Disease

Beneficial Effects of trans-Bis(5-amidino-2-benzimidazolyl)ethene, a Novel, Macrophage-directed Anti-inflammatory Agent

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Previously bis(5-amidino-2-benzimidazolyl)-methane (BABIM) was identified as a strong inhibitor of the multisystem inflammatory disease induced in Lewis rats by injection of streptococcus group A cell wall-derived peptidoglycan polysaccharide (PG-APS). A BABIM derivative, trans-bis(5-amidino-2-benzimidazolyl)ethene (BBE), has attracted attention because of striking qualitative and quantitative differences in its activities when compared with the parent compound. BBE could control destructive tibial osteitis and necrotizing granulomatous splenitis and hepatitis, regardless if given in a preventive or curative mode. The compound had little effect on synovitis, however. BABIM, on the other hand, was active against synovitis and osteitis, but not against splenic granuloma formation. To be effective, it needed to be applied in a preventive mode. BBE caused a characteristic enlargement of PG-APS-laden splenic and hepatic macrophages suggesting that those cells represent targets of the inhibitor. BBE may be a powerful tool for the study of granulomatous lesions. (Am J Pathol 1991, 139:921–931)

The authors previously discovered that bis(5-amidino-2-benzimidazolyl)methane (BABIM) is a powerful anti-inflammatory agent.[1,2] The test model used was the Lewis rat in which a single systemic application of purified streptococcus group-A cell-wall–derived peptidoglycan polysaccharide (PG-APS) leads to early (days 1–3) development of synovitis, destructive osteitis, and granulomatous splenitis and to late appearance (day 9) of granulomatous hepatitis. Uninterrupted daily injections of BABIM, when given onward from day-2 of the experiment, were able to suppress or ameliorate all those responses. The effect on the splenic lesions was only short-lived, however. The inhibitory potency of the compound raised the question whether its mechanism of action might be tied to its other known activity, i.e., its ability to block arginine- and lysine-directed proteases.[3,4] Susceptible enzymes would include incompletely characterized cell-associated trypsinlike proteases, as well as thrombin, plasmin, and urokinase-type plasminogen activator all of which are known to be involved in inflammation.[5–10] To identify such a protease target, 12 additional bis-benzimidazoles have been investigated for their antiinflammatory effectiveness. Besides establishing structure-activity relationships, the study has shown an exciting new compound, trans-bis(5-amidino-2-benzimidazolyl)ethene (BBE). The drug is exceptional in its blockage of granulomatous lesions and its ability to act in a preventive as well as curative mode. Furthermore, it induces highly characteristic morphologic changes in splenic and hepatic macrophages.

Materials and Methods

Synthesis of Benzimidazoles

Table 1 lists the structures and formulas of the compounds tested, and it also includes the inhibition constants (K_i) for trypsin and thrombin.

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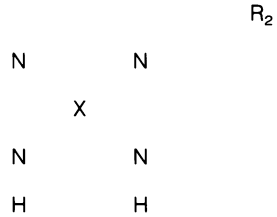
Table 1. *Structure of Benzimidazoles Together with Their Inhibition Constants (K_i) for Bovine Trypsin and Thrombin*



No.	R_1	R_2	X	Formula	K_i (μM) Trypsin	K_i (μM) Thrombin
1 (BABIM)	Am	Am	CH_2	$C_{17}H_{16}N_8 \cdot 4HCl \cdot 2H_2O$	0.017	4.15
2	Am	Am	$(CH_2)_2$	$C_{18}H_{18}N_8 \cdot 4HCl \cdot 2H_2O$	4.7	11.6
3 (BBE)	Am	Am	CH = CH	$C_{18}H_{16}N_8 \cdot 4HCl \cdot H_2O$	5.0	1.6
4	Am	Am	$(CH_2)_3$	$C_{19}H_{20}N_8 \cdot 4HCl \cdot 2H_2O$	27.8	79.0
5	Am	Am	$(CH_2)_4$	$C_{20}H_{22}N_8 \cdot 4HCl \cdot 1.5H_2O$	2.9	14.9
6	Am	Am	$(CH_2)_5$	$C_{21}H_{24}N_8 \cdot C_2H_5OH \cdot 3H_2O$	9.5	9.9
7	Am	Am	$(CH_2)_6$	$C_{22}H_{26}N_8 \cdot 4HNO_3 \cdot 2H_2O$	5.9	4.3
8	Am	H	CH_2	$C_{16}H_{14}N_6 \cdot 3HCl$	0.39	20.7
9	H	H	CH_2	$C_{15}H_{12}N_4 \cdot 2HCl \cdot 0.3H_2O$	>1000	>500
10	CH_2NH_2	CH_2NH_2	CH_2	$C_{17}H_{18}N_6 \cdot 2HCl \cdot 1.6H_2O$	172	>200
11	Im	Im	CH_2	$C_{21}H_{20}N_8 \cdot 2.7HCl \cdot 2.8H_2O$	>1000	>500
12	Im	Im	$(CH_2)_2$	$C_{22}H_{22}N_8 \cdot 4HCl$	617	>1000
13	Im	Im	$(CH_2)_3$	$C_{23}H_{24}N_8 \cdot 4HCl$	>1000	>1000

Am = amidino group, C(NH)(NH2); Im = imidazoline group, C3H3N2H

The synthesis and physical data for compound numbers 1 through 4, 6, 7, and 12 have been described.[3] Compound number 13 was a gift from H. Loewe, Hoechst AG, Germany. Compound numbers 5 and 9 were synthesized according to similar methods as previously described.[3] Compound number 5 was prepared by the condensation of 3,4-diaminobenzamidine hydrochloride with the bis-iminoether obtained from 1,4-dicyanobutane. The purified product, 1,4-bis(5-amidino-2-benzimidazolyl)butane, had a melting point of 265°C. Purity (>98%) and structure were confirmed by elemental analysis (C,H,N), 300 MHz nuclear magnetic resonance spectrometry (NMR) and high-pressure liquid chromatography (HPLC).[11] Compound number 9 was similarly prepared by the condensation of o-phenylenediamine with the bis-iminoether obtained from malononitrile. The purified product, bis(2-benzimidazolyl)methane, had a melting point of 315°C. Purity (>98%) and structure of the product were determined by elemental analysis (C,H,N), HPLC and NMR.

Compound numbers 10 and 11 were synthesized from bis(5-cyano-2-benzimidazolyl)methane. For compound number 10, the starting dinitrile[3] was reduced over platinum oxide at ambient temperature and 40 psi of hydrogen in a Parr hydrogenator to give the desired product, bis(5-aminomethyl-2-benzimidazolyl)methane. Compound number 11 was synthesized by converting the dinitrile to the corresponding diimidate followed by reaction of the intermediate with ethylenediamine to give the desired product, bis(5-imidazolyl-2-benzimidazolyl)methane. The melting points of compound numbers 10 and 11 were 264°C and 277°C, respectively. The purity (>97%) and structures of both compounds were determined by elemental analysis, NMR and HPLC.

Compound number 8 was prepared in three steps, the first one being the condensation of o-phenylenediamine with cyanoacetamide to yield 2-cyanomethylbenzimidazole. The second step was the acid hydrolysis of the nitrile group to give α-(2-benzimidazolyl)acetic acid. The acid was condensed with diaminobenzimidine in polyphosphoric acid to give the desired product, (5-amidino-2-benzimidazolyl)-(2-benzimidazolyl)methane, melting point 285°C. Purity (> 96%) and structural confirmation were determined in the previously described manner.

Streptococcal Cell-wall Fragments

Purified cell walls were prepared by a modification of a previously described method.[12] Disruption of group A, type 3, strain 58 streptococci was carried out for 5 minutes in a Dyno-Mill (Glen Mills, Inc., Maywood, NY) kept at 4°C. The glass beads were removed by coarse filtration, and any remaining intact streptococci were collected by low-speed centrifugation. The cell fragments were obtained by centrifugation for 30 minutes at 30,000 × g. The washed cell walls were extracted three times at 56°C with 2% SDS in phosphate-buffered saline (PBS) of pH 7.0,

washed again repeatedly, first with PBS and then with distilled water, lyophilized, and stored at 4°C.

Before each experiment, the lyophilized cell walls were resuspended in saline and sonicated for 35 minutes at 4°C. The resultant preparation was centrifuged for 30 minutes at 10,000 × g, and the supernatant was passed through a Millipore filter (0.45 μm) to remove any trace of whole bacteria or cell-wall aggregates. This final preparation was kept at 4°C until use. Aseptic techniques were used throughout.

Determination of Inhibition Constants (K_i Values)

K_i values were obtained from published records for previously established inhibitors[3,4] and were newly determined for the remaining compounds. Target enzymes included trypsin and thrombin, and in some instances, also urokinase and plasmin. Conveniently available chromogenic substrates were employed in the assays, and initial reaction velocities at 37°C were plotted according to Dixon[13] to obtain the inhibition constants. Details of the methods have been published.[4,14] In short, all tests were carried out at pH 8.1, except with plasmin in which a pH of 7.6 was chosen. α-N-Benzoyl-DL-arginine-p-nitroanilide (Sigma, St. Louis, MO) was the substrate used with trypsin, thrombin and plasmin, whereas L-pyroglutamyl-glycyl-L-arginine-p-nitroanilide (KABI VITRUM, Stockholm, Sweden) was chosen for urokinase. Lyophilized bovine thrombin was a product of Armour Pharmaceutical Co., Chicago, Illinois. Lyophilized bovine trypsin was obtained from Sigma, St. Louis, Missouri, human plasmin was obtained from KABI VITRUM, Stockholm, Sweden, and human urokinase (Abbokinase) was received from Abbott Laboratories, North Chicago, Illinois. The enzymatic release of p-nitroaniline was followed spectrophotometrically. All determinations were carried out in triplicate.

Animals

Female Lewis rats (Charles River Breeding Colonies, Wilmington, MA) with an average body weight of 145 g were used for all animal experiments under specific pathogen-free conditions. They were kept on an unrestricted diet of laboratory chow and water. Disease was initiated by a single intraperitoneal injection of 0.5 ml of the PG-APS suspension described earlier. The amount of streptococcal cell-wall material was equal to 15 μg of rhamnose equivalent per gram of body weight. Control animals received PBS only. Treatment with inhibitor involved single daily injections of the test agent for the time period specified. The drug was dissolved in 0.5 ml pyrogen-free saline and was injected into the tail vein under light ether anesthesia.

Histologic Examination and Scoring of the Lesions

The animals were killed with an overdose of carbon dioxide. Spleen and liver were expeditiously removed and weighed, and pieces of both organs were submitted for histologic evaluation. The hind legs were amputated through the mid femur, skinned, fixed, decalcified, and then divided into a proximal piece containing the knee joint and proximal tibia and a distal piece including ankle and foot joints. All tissues were stained with H&E and with periodic acid Schiff (PAS) after diastase digestion. The severity of the synovitis in ankle and knee joints was judged separately on a scale of 0 to 4 according to the density of the inflammatory infiltrate, the degree of synovial-lining–cell hyperplasia and the amount of intraarticular exudate. For each animal, only one extremity was rated and the values of knee and ankle joints were combined for the total synovitis score. The extent of the granulomatous disease in the spleen was ranked by one of two systems. One, which was designed for early disease and identified as "granuloma score," considered the number of granulomata as well as the size of the area of destruction, whereas the other, which was designed for late disease, was based only on the area of parenchymal replacement. The number of granulomata in the liver per given area was determined with the help of a Zeiss Videoplan computer image analyzer (Carl Zeiss, Germany). The osteitis in the distal tibial metaphysis and epiphysis was graded on a scale of 0 to 4 taking into consideration the density of the intraosseous inflammatory infiltrate and the degree of destruction of medullary and cortical bone and of the epiphyseal and joint cartilage. The reparative bone score, from 0 to 4, is an expression of the degree of healing of the distal tibial defect by newly formed trabecular bone.

Statistical Analysis

Student's *t*-test was employed to evaluate the statistical significance of the difference between two means.

Results

Structure–Activity Relationships

In this study, 12 additional BABIM-related bisbenzimidazoles were evaluated for their ability to sup-

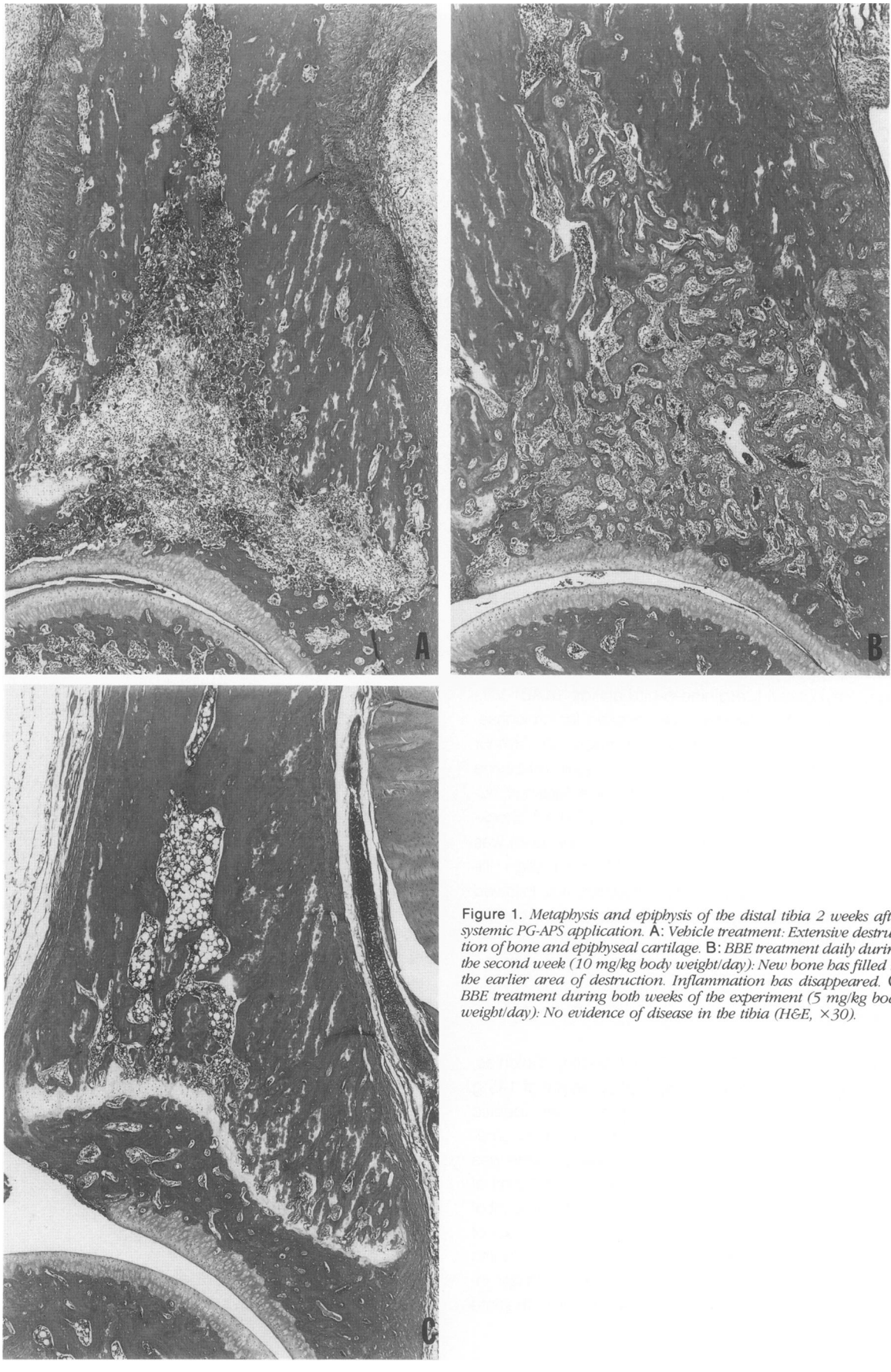


Figure 1. *Metaphysis and epiphysis of the distal tibia 2 weeks after systemic PG-APS application.* A: *Vehicle treatment: Extensive destruction of bone and epiphyseal cartilage.* B: *BBE treatment daily during the second week (10 mg/kg body weight/day): New bone has filled in the earlier area of destruction. Inflammation has disappeared.* C: *BBE treatment during both weeks of the experiment (5 mg/kg body weight/day): No evidence of disease in the tibia (H&E, ×30).*

press PG-APS-induced disease. The test animals received five consecutive daily injections of inhibitor. The first dose was given immediately before the streptococcal cell-wall fragments and the last dose was given 24 hours before sacrifice. In previous experiments, 5-day period were long enough for the rats to develop synovitis, tibial osteitis, and granulomatous disease in the spleen. The only important facet that could not be studied under this time restraint was granulomatous hepatitis, which would appear about 4 days later.[2] The daily dose of inhibitor was set at 10 mg/kg body weight, except in those cases in which the amount had to be reduced because of solubility problems. In each separate experiment, one to four of the compounds were examined simultaneously. Three types of controls were always included: animals that had received neither PG-APS nor inhibitor; animals that had received PG-APS, but no inhibitor; and animals that had received PG-APS and BABIM. The latter two controls ensured the reproducibility of the responses. Toxicity was also evaluated for each inhibitor. The individual groups were made up of six animals, except for the first type of control where n was four.

As can be seen from their molecular structures in Table 1, all inhibitors shared the presence of two benzimidazole moieties with BABIM. Variations from the parent compound resulted from modification of the central linking chain and/or replacement of one or both amidino groups. Table 2 contains the data on the ability of the compounds to reduce synovitis, destructive osteitis in the distal tibia, and splenic disease. BABIM was effective in blocking the first and second response, but showed no significant amelioration of the third. In previous studies, BABIM had caused a mild reduction in granulomatous splenitis on day 5,[2] but this variance with the current findings is explained by the fact that BABIM treatment in those cases was begun 2 days before PG-APS injection.

Four compounds (nos. 4–7), which differed from BABIM only by the length of their central carbon chain, followed the same general pattern of inhibition as BABIM, although they did not match its potency. The two-carbon chain derivative, no. 2, was able not only to reduce synovitis and osteitis, but also to diminish splenic enlargement and granuloma formation. Compounds no. 10 and 12 were distinguished chiefly by their reductive influence on splenic weight which histologically was the result of a decrease of the immune response. Compound no. 3 (BBE) finally, the ethene derivative of BABIM, stood out by its ability to achieve the greatest reduction in spleen weight and to block completely any granulomatous response in that organ. BBE also effected a modest decrease in osteitis, but did not alter the severity of the synovitis.

A comparison of the antiproteolytic potency of the compounds, as reflected by the K_i values in Table 1, with the anti-synovitis or anti-granuloma activity, as registered in Table 2, did not show any correlation. Compound 4, for example, could ameliorate synovitis, whereas number 8 was devoid of such activity despite possessing lower K_i values than number 4 against trypsin as well as thrombin. A similar case can be made against a role for trypsin and thrombin inhibitors in granulomatous splenitis. Thus, number 2 is a weaker inhibitor than number 1 for trypsin and thrombin, but still outranked the latter in its ability to control splenitis.

Table 2. *Inhibitory Effect of Benzimidazoles on PG-APS-induced Synovitis, Tibial Osteitis, and Splenic Enlargement and Granulomatous Disease*

Compound no.	Synovitis score 0–8	Tibial osteitis score 0–4	Spleen	
			Weight* mg/100g b.w.	Granuloma score 0–4
No inhibitor	3.21 ± 1.15	1.76 ± 0.95	770 ± 78	2.13 ± 0.52
1 (BABIM)	0.67 ± 0.83	0.04 ± 0.07	646 ± 133	2.27 ± 1.01
2†	1.48 ± 0.96	0.27 ± 0.2	594 ± 55	0.90 ± 0.5
3 (BBE)	3.50 ± 0.71	0.71 ± 0.81	398 ± 17	0
4	1.14 ± 0.93	1.27 ± 0.96	740 ± 140	1.77 ± 0.41
5†	0.88 ± 0.52	0.75 ± 0.87	743 ± 43	2.00 ± 0
6	1.13 ± 0.86	0.75 ± 0.86	640 ± 114	1.92 ± 0.8
7†	0.83 ± 1.81	0.25 ± 0.61	603 ± 73	2.02 ± 1.23
8	3.38 ± 1.05	1.17 ± 0.82	690 ± 169	1.88 ± 1.08
9	2.92 ± 1.93	1.42 ± 1.56	602 ± 116	1.43 ± 0.82
10	1.71 ± 0.87	0.33 ± 0.52	447 ± 77	1.15 ± 0.95
11	3.00 ± 0.40	1.00 ± 1.55	709 ± 138	1.78 ± 0.53
12	2.33 ± 0.61	0.92 ± 1.43	555 ± 56	1.35 ± 0.70
13	3.00 ± 1.95	0.33 ± 0.82	673 ± 1.04	2.20 ± 1.10

* In 24 rats that had received neither PG-APS nor inhibitor the spleen weight was 235 ± 17 mg/100 g body weight.
† Drug dose reduced to 7.5 mg/100g body weight.
There were 42 rats in the control groups which had received only PG-APS. There were 41 rats in the groups which had received BABIM as well as PG-APS. All other groups were made up of six animals. Values are means ± SD.

Comparison of BABIM and BBE

From the studies just discussed, BABIM and BBE emerged at opposite ends of the spectrum of inhibitory activities. BABIM excelled as inhibitor of synovitis, but was ineffective in controlling granulomatous splenitis. The converse was true for BBE. There was a certain sharing of common ground in that both compounds were able to reduce the severity of the osteitis. To further delineate their behavior and possible usefulness, a series of experiments was carried out.

To judge the absolute potency of BBE, the same 5-day test used to obtain the data for Table 2 was repeated with reduced amounts of BBE. As shown in Table 3, a 5-mg dose completely suppressed granuloma formation in the spleen, and even a 2.5-mg dose had a significant effect. BABIM again did not alter the splenitis, although it produced a mild reduction in spleen weight.

In the next series of experiments, the duration was extended to 14 days. This was done to judge the effect of the inhibitors on granulomatous hepatitis, a lesion that begins to develop approximately 9 days after PG-APS injection.[2] In one set of animals, treatment was in a curative fashion, i.e., the disease was allowed to proceed for 1 week, and then either BABIM or BBE were given during the second week. In another group of animals, treatment with BBE was in a preventive mode, i.e., it was started at the same time that PG-APS was administered. The influence on the joint and bone changes is presented in Table 4, whereas the findings in liver and spleen are incorporated in Table 5.

From the data in Table 4, it is evident that at the time of sacrifice, the control animals had severe synovitis and osteitis. The distal tibial metaphysis and epiphysis had lost all trabecular bone in a process of neutrophil-assisted osteoclasia,[2] and the hollowed-out space was filled in by granulation tissue (Figure 1A). The epiphyseal plate was fragmented or missing. In animals that had received BABIM during the second week, there was a modest lessening of the synovitis and osteitis, and an abortive attempt at healing in the epiphysis and metaphysis as indicated by early new bone formation (rated as Reparative Bone Score) occurred. In the group that had been supplied with BBE during the second week, there was no improvement in the severity of the synovitis, but there was clearing of all active osteitis and the metaphyseal/epiphyseal defects had already been completely filled in with new trabecular bone (Figure 1B). In the treatment group that had been given BBE throughout both weeks, there was some improvement in the synovitis, but more impressive was the total absence of any lesion in the bone and thus also of the need for a reparative process (Figure 1C).

The data on liver and spleen for the animals just discussed are also instructive (Table 5). In the vehicle-treated controls, PG-APS application brought about a 2.4-fold increase in liver weight and a 7-fold increase in spleen weight as compared with non-PG-APS-treated controls (footnote, Table 5). In the liver parenchyma numerous macrogranulomata were present. They are defined as lesions whose diameter exceeds [fr ½] high-power field at × 450. The majority of the granulomata contained central collections of neutrophils often associated with necrosis (Figure 2A). In the spleen of these control animals, there were large confluent granulomatous lesions with a geographic pattern (Figure 2B). More than two-thirds of the parenchyma was thus afflicted. As can be seen in Table 5, BABIM treatment during the second week did not significantly alter any of the liver or spleen parameters studied. BBE treatment during the same period, however, efficiently limited weight gain of liver and spleen. There was a reduction in the number of hepatic macrogranulomata, which was statistically significant if the organ-weight differential against the controls was taken into consideration. The size of the area of necrosis and granulomatous inflammation in the spleen was held to 11.5% compared with 68.3% in the controls. The 2-week course of BBE application also controlled organ

Table 3. *Effect of Reduced Doses of BBE on Synovitis, Tibial Osteitis, and Spleen Weight and Splenic Granulomatous Inflammation*

Treatment*				Spleen†	
Inhibitor	Daily dose mg/kg b.w.	Synovitis score 0–8	Tibial osteitis score 0–4	Weight mg/100 g b.w.	Granuloma score 0–4
None	—	2.63 ± 0.59	1.42 ± 0.86	816 ± 48	1.93 ± 0.59
BABIM	10	0.38 ± 0.47‡	0	670 ± 105‖	2.48 ± 1.03
BBE	5	2.75 ± 0.63	0.63 ± 0.97	428 ± 32‖	0
BBE	2.5	2.83 ± 0.88	0.87 ± 0.71	560 ± 67‡	0.57 ± 0.59¶

* Groups of six animals each received PG-APS and five consecutive daily doses of inhibitor in the amounts stated. The animals were sacrificed 1 day after the last injection. All values are means ± SD.

† The spleen weight of rats that had received neither PG-APS nor inhibitor was 232 ± 8 mg/100 g b.w. (n = 4).

‡ $P < 0.0001$ against vehicle-treated controls.

‖ $P < 0.05$ against vehicle-treated controls.

¶ $P < 0.005$ against vehicle-treated controls.

Table 4. *Synovitis, Tibial Osteitis, and Reparative New Bone Formation 2 Weeks after PG-APS Injection: Effect of Delayed Treatment with Either BABIM or BBE and of Uninterrupted* ab initio *Treatment with BBE*

Treatment*			Tibia		
	Daily dose mg/kg b.w.				
Inhibitor	1st week	2nd week	Synovitis score 0–8	Osteitis score 0–4	Reparative bone score† 0–4
None	—	—	2.60 ± 1.12	3.30 ± 0.27	0
BABIM	—	10	1.83 ± 0.90	2.25 ± 1.70	0.50 ± 0.84
BBE	—	10	2.97 ± 0.72	0.08 ± 0.20‡	4
BBE	5	5	1.43 ± 1.11	0	0

* n = 6 for all groups. Values are means ± SD.
† Degree of healing by new bone formation.
‡ $P < 0.001$ against vehicle-treated controls.

weights and prevented appearance of any macrogranulomata in the liver and of any necrotic lesions in the spleen. A surprise finding, however, was the presence of numerous hepatic and splenic microgranulomata in both BBE series (Figure 2C, D). They will be further discussed in the following section.

BBE-induced Changes in Macrophage Morphology

After its injection into the peritoneal cavity, PG-APS is taken up by macrophages that rapidly gain access to the general circulation. Within a few hours those cells can be identified in the ramifications of the portal venous system, and the streptococcal cell-wall fragments will appear increasingly in the Kupffer cells. At the same time, noticeable accumulation of the material in splenic macrophages occurs, initially mainly in the marginal zone, but later on also in the periphery of the red pulp. In animals that have received PG-APS only or PG-APS as well as BABIM, the PAS-positive deposits in the macrophages in liver and spleen appear in finely granular form (Figure 3A, B). In animals that have been treated with BBE, however, the macrophages in both organs take up excessive amounts of PG-APS. The cells become massively enlarged and the granules fuse (Figure 3C, D). The abundance of the PAS-positive deposits imparts a glossy, lacquered quality to the cytoplasm. In animals that have been treated with BBE for 14 days, but less so in those treated for only 7 days, the overstuffed hepatic and splenic macrophages appear to have a tendency to burst and release some of the PG-APS. This, in turn, leads to attraction of monocytes from the circulation which ingest the material and aggregate in groups, considerably smaller than the macrogranulomata (Figure 2C, D).

The unexpected discovery of the microgranulomata—by definition lesions with a diameter of less than [fr ½] high power field at × 450—in animals still under BBE protection raised the question if discontinuation of treatment would cause transformation of the aggregates into full-blown macrogranulomata with accompanying neutrophilic infiltration and necrosis. Similarly, one might wonder about a possible late recrudescence of the joint and bone lesions. To obtain an answer, rats were given daily injections of either BABIM or BBE for 1 week and were then allowed to survive for 18 additional days without further treatment. The results are summarized in Table 6 and Table 7, for synovium and bone, and liver and spleen, respectively. In the drug-free controls, active synovitis and severe destructive osteitis with little evidence of

Table 5. *Hepatic and Splenic Granulomatous Disease 2 Weeks after PG-APS Injection: Effect of Delayed Treatment with BABIM or BBE and of Uninterrupted* ab initio *Treatment with BBE*

Treatment*			Liver†			Spleen‡	
	Daily dose (mg/kg b.w.)						
Inhibitor	1st week	2nd week	Weight mg/100 g b.w.	Macro-granulomata no./100 mm²	Micro-granulomata no./100 mm²	Weight mg/100 g b.w.	Necrosis % of cut surface
None	—	—	9,572 ± 1,575	58 ± 41	77 ± 13	1,756 ± 256	68.3 ± 8.7
BABIM	—	10	9,158 ± 1,485	52 ± 34	63 ± 27	1,484 ± 259	63.3 ± 9.8
BBE	—	10	6,087 ± 581‖	24 ± 31	208 ± 24	520 ± 160‖	11.5 ± 10.5‖
BBE	5	5	5,561 ± 622‖	0	926 ± 266	755 ± 157‖	0

* n = 6 for all groups. Values are means ± SD.
† The liver weight of rats that had received neither PG-APS nor inhibitor was 3,931 ± 281 mg/100 g b.w. (n = 4).
‡ The spleen weight of rats that had received neither PG-APS nor inhibitor was 250 ± 45 mg/100 g b.w. (n = 4).
‖ $P < 0.0005$ against vehicle-treated controls.

Table 6. *Synovitis, Tibial Osteitis, and Reparative New Bone Formation 25 Days after PG-APS Injection: Effect of Initial 1-Week Treatment with either BABIM or BBE*

Treatment*			Tibia	
Inhibitor	Daily dose mg/kg b.w.	Synovitis score 0–8	Osteitis score 0–4	Reparative bone score 0–4
None	—	2.68 ± 1.08	3.44 ± 0.61	1.06 ± 1.05
BABIM	10	1.74 ± 1.40	2.75 ± 1.00	0.69 ± 1.03
BBE	5	0.89 ± 1.28†	0	0

* n = 8 for all groups. Values are means ± SD.
† $P < 0.05$ against vehicle-treated controls.

bone repair occurred. Liver weights and extent of granulomatous involvement were about the same as at the end of the earlier 2-week experiment (Table 5). However, healing of the granulomata by scarring had begun. The spleens in these controls were considerably smaller than at the 2-week mark because of removal of much of the necrotic debris. All viable splenic red pulp was densely packed with trilineage hemopoietic elements. A glance at the data for BABIM shows that the compound caused slight improvement in synovitis and osteitis (Table 6), but had no effect at all on liver and spleen (Table 7). BBE application, on the other hand, led to maintenance of a significant suppression of synovitis, preserved the integrity of the tibial bone, and almost completely protected liver and spleen. Even after the 18-day drugfree period, the livers were without macrogranulomata, and the microgranulomata were small in number. As to the spleen, the histologic appearance was normal, i.e., there were neither granulomata nor hemopoietic elements.

Finally, the distribution of PG-APS in the macrophages can be altered secondarily. In the experiment in which the rats received BBE only in the second week, for example, the intracytoplasmic deposits in the macrophages during the first week would have been in the finely granular form. However, when the animals were killed after the second week, the macrophages in liver and spleen showed the typical BBE effect, i.e., massive enlargement.

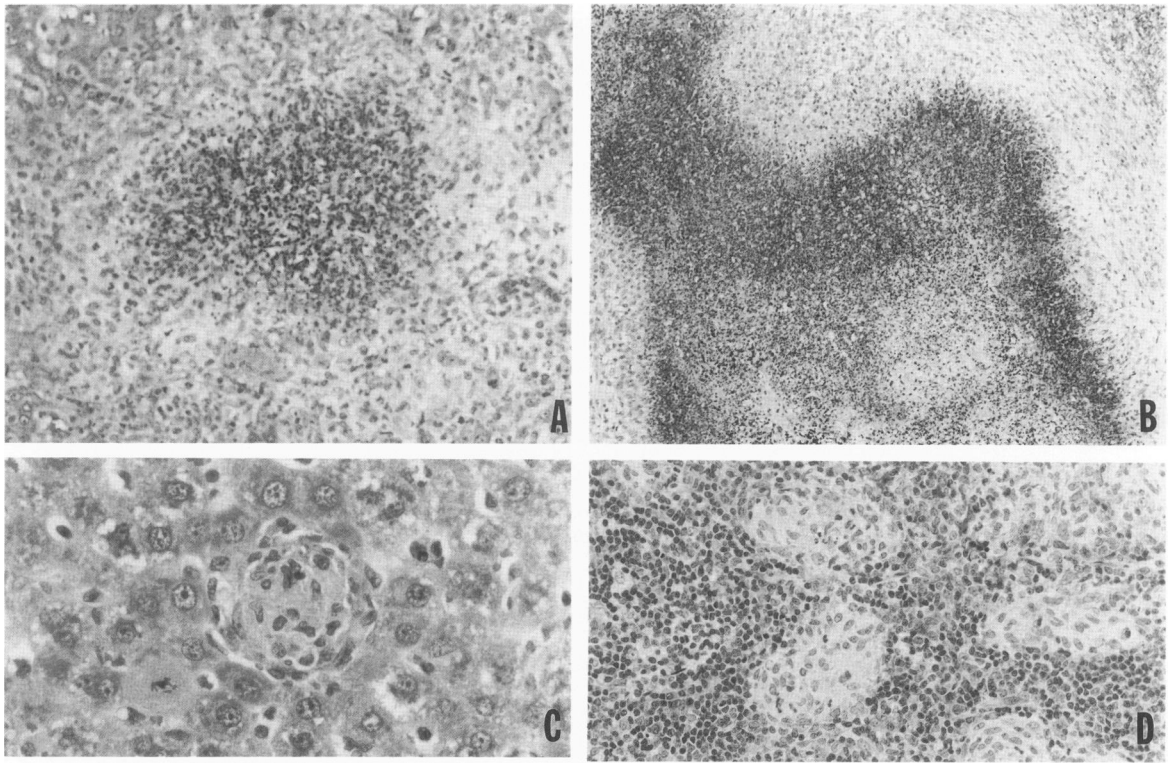


Figure 2. *Sections of rat liver (A,C) and spleen (B,D) two weeks after systemic injection of PG-APS.* Vehicle-treated rats: *A. Hepatic macrogranuloma with central necrosis and neutrophilic infiltrate. B. Confluent necrotizing granulomatous inflammation in the spleen.* BBE-treated rats (5 mg/kg body weight/day): *C. Hepatic microgranuloma without necrosis or neutrophils. D. Splenic microgranulomata without necrosis or neutrophils (H&E; A and D, ×175; B, ×88; C, ×350).*

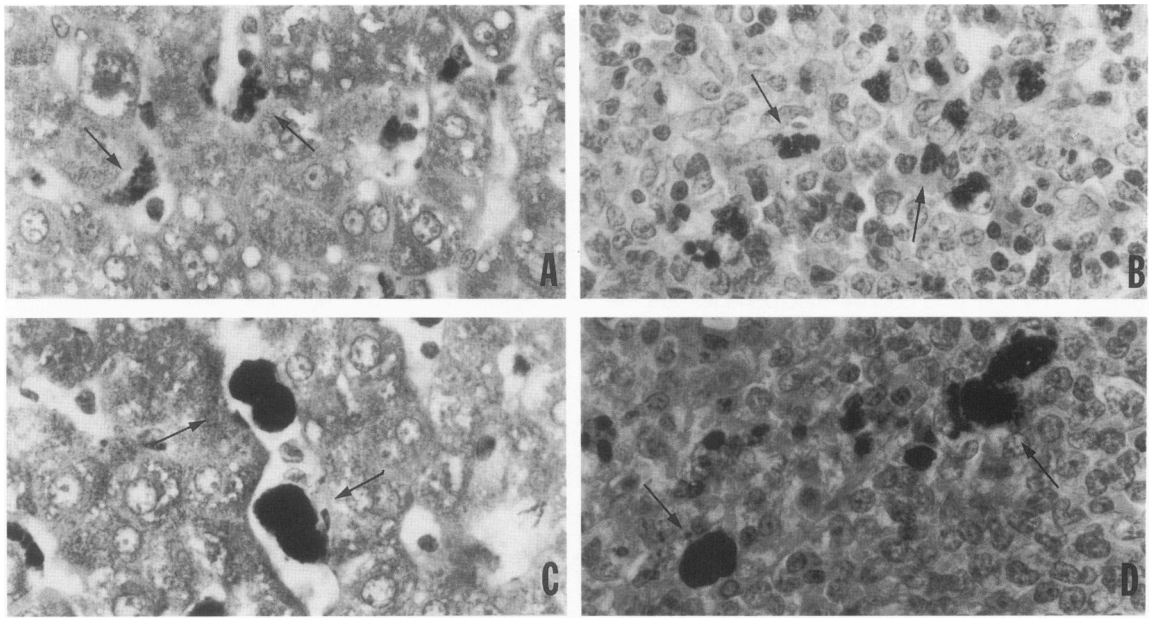


Figure 3. *Streptococcal cell wall deposits in rat Kupffer cells (A,C) and splenic macrophages (B,D) 5 days after systemic PG-APS injection. A,B: Small size of Kupffer cells and splenic macrophages, respectively, in vehicle-treated animals. C,D: Massive enlargement of Kupffer cells and splenic macrophages, respectively, in animals which had received BBE in the daily dose of 10 mg/kg body weight. Arrows point towards the Kupffer cells and splenic macrophages, respectively. (PAS stain, ×525).*

Discussion

Study of the antiinflammatory properties of BABIM derivatives has revealed not only a significant variation in potency of the individual compounds but also a surprising qualitative difference in the inhibitory spectrum. At one end, there is BABIM, which is active chiefly against synovitis and tibial osteitis and is most effective in a preventive role. At the other end, there is BBE, which excels in the suppression of splenic and hepatic granulomatous disease and also of tibial osteitis, and which is able to act in a preventive as well as curative mode. The divergent behavior of the compounds argues for different points of interaction, i.e., interference with an early reaction sequence for BABIM and blockage of a continually ongoing reaction for BBE. The effectiveness of BABIM when given from the beginning of the disease and its failure when started late, cannot be attributed to the more acute nature of the inflammation in the early stages and its more chronic nature in the late stages, because when given preventively without interruption, the compound has been effective in acute as well as chronic synovitis.

The histologic changes in splenic and hepatic macrophages clearly single out those cells as targets of BBE, and so far all other compounds with the ability to block splenic granuloma formation have shown a similar proclivity to cause macrophage enlargement. However, increased storage of PG-APS itself is not the proximate cause of the antigranulomatous effectiveness of a given compound. This is known because several benzimidazoles, such as numbers 5 and 7, produce bloating of the cells but do not inhibit granuloma formation.

Table 7. *Liver and Spleen Weights and Hepatic Granulomata 25 Days after PG-APS Injection: Effect of Initial 1-Week Treatment with either BABIM or BBE*

Treatment*		Liver†			Spleen†
Inhibitor	Daily dose mg/kg b.w.	Weight mg/100 g b.w.	Macrogranulomata no./100 mm^2	Microgranulomata no./100 mm^2	Weight mg/100 g b.w.
None	—	10,778 ± 914	68 ± 15	4 ± 2	629 ± 153
BABIM	10	9,662 ± 1,235	60 ± 22	4 ± 3	643 ± 444
BEE	5	4,475 ± 219‡	0	49 ± 12	302 ± 23‡

* n = 8 for all groups. Values are means ± SD.

† The liver and spleen weights in rats that had received neither PG-APS nor inhibitor were 3,893 ± 66 mg/100 g b.w. and 204 ± 18 mg/100 g b.w., respectively (n = 4).

‡ $P < 0.0001$ against vehicle-treated controls.

Currently, how BBE might stimulate the massive deposition of PG-APS in splenic and hepatic macrophages is uncertain. Accelerated production of cell-surface receptors, enhanced numbers of phagosomes, impedance of phagolysosome fusion, and a reduction of the release of stored material are mechanisms to be considered. Electron microscopic methods are being employed to investigate those possibilities. The appearance of bloated macrophages is also influenced by the microenvironment. Though PAS-positive macrophages are encountered in the inflamed synovium and tibial marrow, no giant forms have been identified in those locations.

It has been shown that the microgranulomatous reaction to rupture of PG-APS-laden macrophages in BBE-treated animals does not proceed to a macrogranulomatous response, even if treatment is halted. There are at least two possible explanations for such a favorable outcome. First, the ingested PG-APS might be so modified within the macrophages that, when released, it does not evoke the complete macrogranulomatous event. Second, BBE might be retained in the tissues or macrophages for a prolonged period of time and might thus exert a continuous restraining influence on the inflammatory process. Although the second hypothesis is favored at this point, detailed pharmacokinetic investigations will be needed to prove its validity. Regardless of the outcome of such a study, it is believed that microgranulomata in the aftermath of BBE treatment will generally heal without permanent damage.

In view of the ability of BABIM and its congeners to inhibit trypsinlike enzymes, blockage of such proteases might account for the beneficial effect of the compounds on PG-APS-induced inflammation. However attractive the idea was, it could not be supported by the findings of this study. The data in Tables 1 and 2, as discussed in the Results section, have already removed trypsin and thrombin from consideration as targets. This brings up urokinase-type plasminogen activator (UK) as another likely candidate for inhibition. UK is a secretory product of stimulated macrophages.[15,16] It transforms plasminogen into plasmin which in turn is able to hydrolyze fibrin, to split a variety of proteins, such as basement membrane components, to stimulate release of interleukin 1 from monocytes, and to activate other enzymes, including procollagenase.[8–10,17] UK thus plays a prominent role in acute and chronic inflammation. If the antigranulomatous effect of BBE were directed against UK, its K_i value for the enzyme should have been lower than with BABIM. In fact, however, the converse was true BBE having a Ki value of 1.15×10^{-5} M and BABIM of 2.33×10^{-6} M. UK inhibition could also not be tied to antisynovitis potency. This derives from the fact that compound number 7 and compound number 8 had nearly identical K_i values against UK (1.42×10^{-4} M and 1.72×10^{-4} M, respectively), and still the former compound was a competent inhibitor of synovitis, whereas the latter was completely ineffective. Similar arguments can be brought forward against plasmin inhibition playing a role in successful treatment. The antigranuloma agent BBE again was a weaker inhibitor than the antisynovitis agent BABIM (K_i 7×10^{-5} M versus 2.65×10^{-6} M, respectively), and the potent antisynovitis agent number 2 had a K_i value not greatly different from the inactive agent number 8 (3.92×10^{-5} M versus 5.23×10^{-5} M, respectively).

In several respects, the immunosuppressive agent cyclosporine A (CsA) has a similar effect as BBE on PG-APS-induced disease. It completely prevents granuloma formation in the liver and reduces the severity of the chronic arthritis, but it has no apparent influence on the acute phase of the joint inflammation.[18] Those findings raise the question if the two drugs might not also share the same mode of action. Since CsA has well-defined immunoregulatory properties involving T lymphocytes and macrophages,[19–20] BBE could be tested for those same characteristics to establish or to disprove a functional relationship between the two agents.

At this point, it is undetermined if the power of BBE to suppress granulomatous reactions is restricted to PG-APS-induced lesions or if it also applies to granulomata from other causes. Should the latter be true, the compound might be considered for treatment of such immunologically mediated, noninfectious granulomatous conditions as sarcoidosis and Crohn's disease.

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