Biochemical Pharmacology, Vol. 35, No. 6, pp. 917–922, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

INHIBITION OF RAT NEUTROPHIL FUNCTIONAL RESPONSES BY AZAPROPAZONE, AN ANTI-GOUT DRUG

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(*Received* 13 *May* 1985; *accepted* 6 *August* 1985)

Abstract—Azapropazone at concentrations of 0.1 to 1 mM inhibited by 30–70% rat neutrophil migration, aggregation, and degranulation in response to the synthetic chemotactic peptide fMet-Leu-Phe. Binding studies using fNle-Leu-[^{3}H]Phe, a radiolabeled analog of fMet-Leu-Phe, showed that azapropazone did not inhibit these responses by interfering with fMet-Leu-Phe binding. Azapropazone also decreased both the apparent rate of production and maximal levels of superoxide anion (O_2^-) generated by cells stimulated with 100 ng/ml phorbol-12-myristate-13-acetate (PMA). The concentrations of azapropazone that inhibit these neutrophil responses *in vitro* approximate those previously found *in vivo* after administration of therapeutic doses of drug to rats or humans. Taken together, the data suggest that the efficacy of azapropazone in gouty arthritis may be partly due to its ability to inhibit key neutrophil functional responses *in vivo*.

Azapropazone (5-dimethylamino 9-methyl-2-propyl-1-*H*-pyrazolo[1, 2-*a*][1, 2, 4]benzotriazine-1, 3(2*H*)-dione dihydrate) is a nonsteroidal anti-inflammatory drug (NSAID) first developed by Siegfried A. G. in the early 1960s and marketed now in Europe as Prolixan [1]. In animals and in various human arthritides, azapropazone has been shown to be effective at inhibiting inflammation but at doses 3- to 50-fold higher than other NSAIDs such as indomethacin or phenylbutazone [1, 2]. In gouty arthritis, however, where inflammation results from deposition of uric acid crystals in joints and tissues, azapropazone has greater therapeutic potential compared to other NSAIDs since it not only has anti-inflammatory activity but also inhibits production and enhances the renal tubular excretion of uric acid [3]. These latter properties lead to a significant reduction in both serum urate concentrations and crystal deposition and thereby contribute to remission of the disease.

There is reasonable evidence indicating that the etiology of gouty arthritis is the development of an acute neutrophil-mediated inflammatory response to urate crystals [reviewed in Ref. 4]. Spilberg *et al.* [5] have reported that phagocytosis of urate crystals by synovial polymorphonuclear leucocytes (PMN) stimulates the release of a chemotactic substance that leads to an additional accumulation of inflammatory cells in the articular spaces. Schumacher and Phelps [6], as well as others [7], have cited evidence indicating that urate crystals are toxic to PMN and their ingestion leads to cell lysis and the nonspecific release of lysosomal enzymes. Since PMN granules contain a variety of proteases and other proinflammatory substances which can cause tissue and joint destruction [8], the toxic effects of urate crystals on the PMN may lead to a further augmentation of the inflammatory process.

In view of the important pathological role neutrophils play in gouty arthritis, we have investigated the effects of azapropazone on several different neutrophil *in vitro* functional responses which parallel their *in vivo* functions in inflammation. Our findings suggest that the therapeutic efficacy of azapropazone in acute gouty arthritis may be related to its ability to inhibit a variety of neutrophil functions *in vivo*.

METHODS

Male Crl:CD(SD)Br rats (Charles River Breeding Laboratories, Kingston, NY) weighing 200–250 g were used. Rat peritoneal neutrophils (PMN) were collected 4–6 hr after i.p. injection of 10% (w/v) sodium caseinate (Sigma) and washed several times with Hanks' buffer (pH 7.2) at 4° to remove residual exudate fluids. Cells obtained in this manner are more than 90% PMN as determined by Wright stain and microscopic examination. In experiments where PMN were exposed to drug, cell viability was monitored by measuring release of the cytoplasmic enzyme lactate dehydrogenase (LDH) [9]. In all experiments in this report, the amount of LDH released by both untreated and drug-treated PMN was less than 5% of the total, suggesting an absence of drug cytotoxicity.

Stimuli and drugs. As a stimulus of *in vitro* PMN migration, degranulation and aggregation, the synthetic chemotactic peptide, fmethionyl-leucyl-phenylalanine (fMLP) (Sigma Chemical Co., St. Louis, MP) was used. As a stimulus of PMN superoxide anion (O_2^-) generation, phorbol-12-myristate-13-acetate (PMA) (Sigma) was used. Stock solutions of fMLP (10^{-2} M) and PMA (1 mg/ml) were made

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up in 100% dimethyl sulfoxide (DMSO) with subsequent dilutions being made in Hanks' buffer. In the aggregation and enzyme release assays, the final concentration of fMLP used was 10^{-7} M, and in the migration assay 3.3×10^{-9} M fMLP was used. In the O_2^- assay, a final concentration of 100 ng/ml PMA was used to stimulate. In unreported studies, these concentrations of stimuli were found to elicit maximal PMN responses in the various assays. Stock solutions of azapropazone (Siegfried A. G.) were made up in 100% DMSO with subsequent dilutions being made in Hanks' buffer. In all experiments, the final DMSO concentration did not exceed 0.1% (v/v) and this concentration had no detectable effect on rat PMN viability or biological responsiveness.

Enzyme release assay. Release of the azurophilic granule marker, β-glucuronidase, was measured as an indicator of rat PMN secretory responsiveness. PMN (5×10^6 cells/ml) were preincubated with or without azapropazone for 10 min at 37°, and then cytochalasin B (5 μg/ml) and fMLP (10^{-7} M) were added. The mixture was incubated for 10 min at 37° to facilitate β-glucuronidase release and then chilled at 4° to stop the response. Cells were pelleted by centrifugation (200 *g*, 4°), and aliquots of supernatant fluid were removed and assayed for β-glucuronidase activity. Enzyme activity was assayed by using phenolpthalein glucuronide (Sigma) as a substrate and measuring liberated phenolpthalein spectrophotometrically at 488 nm [10]. Data were collected as mean values of triplicate samples, and the results were calculated as the percent enzyme released relative to values obtained with untreated cells stimulated with 10^{-7} M fMLP.

Aggregation assay. PMN (5×10^6 cells/ml) in Hanks' buffer supplemented with 0.7 mM Mg^{2+} were incubated with or without azapropazone for 10 min at 37°, and then aliquots were transferred to siliconized aggregometer cuvettes with magnetic stirring bars. The cuvettes were placed in a 4 channel aggregometer (Bio/Data, model PAP4) at 37° and stirred constantly at 700 rpm. In all experiments, PPP (platelet poor preparation) values were set at 50% of the experimental cell concentration (i.e. 2.5×10^6 cells/ml). Five microliters fMLP (final concentration = 10^{-7} M) was added to the cells, and the increase in light transmission (ΔT) resulting from cell aggregate formation during a 5-min period was measured. Data were collected in duplicate and are expressed as mean values of six separate experiments plus or minus the standard error.

Superoxide anion (O_2^-) release assay. Superoxide anion (O_2^-) generation was assayed by measuring the O_2^- dependent reduction of ferricytochrome *c* to ferrocytochrome *c* essentially as described by Babior *et al.* [11]. Briefly, 5×10^6 PMN/ml were pretreated with or without various concentrations of azapropazone for 10 min at 37°. Non-reduced cytochrome *c* (0.23 mM) and PMA (100 ng/ml) were added, and the mixture was quickly transferred to quartz cuvettes in a Beckman Du-6 spectrophotometer at 37°. The reduction of cytochrome *c* was monitored as an increase in absorbance (Abs) at 550 nm over a 30-min period. To calculate specific O_2^- reduced cytochrome *c*, the change in Abs at 550 nm detected in the presence of 30 μg/ml superoxide dismutase (SOD) was also measured. Data were converted to nanomoles of O_2^- reduced cytochrome *c* by first subtracting the SOD values and then dividing by the absorbance coefficient for reduced cytochrome *c* ($21.2\ mM^{-1}\ cm^{-1}$) as first described by Van Gelder and Slater [12] and more recently by Goldstein [13]. Rates of O_2^- production were calculated by linear regression analysis of the O_2^- production versus time curve at times between 0 and 15 min. All data are expressed as mean values of three or more separate experiments.

Migration assay. Rat PMN migration in response to fMLP (3.3 nM) was measured in modified Boyden chambers essentially as described by Becker and Showell [14]. Briefly, 10^6 PMN/ml suspended in Hanks' buffer supplemented with 1 mg/ml bovine serum albumin (Sigma) and 0.7 mM Mg^{2+} (final concentration) were preincubated with or without azapropazone for 10 min at 37°. The cells (200 μl) were added to the upper wells of Boyden chambers separated from bottom wells and chemoattractant (110 μl of 3.3 nM fMLP) by 3.0 μm pore size MF Millipore filters (Millipore, Bedford, MA). The chambers were then incubated in humidified containers for 45 min at 37°. After incubation the filters were removed, stained with hematoxylin, and mounted on slides with Permount (Sigma) for counting. Cell migration was measured by the leading front technique as reviewed by Wilkinson [15], and data are expressed as mean values of five determinations from duplicate filters plus or minus the standard error of the mean. The leading front was defined as the distance into filter (microns) where 3–5 stained cells were clearly visible.

Binding assay. Rat PMN were incubated with or without azapropazone for 10 min at 37° and then assayed for fNle-Leu-[^{3}H]Phe ([^{3}H]fNLLP) binding activity as described by Mackin *et al.* [16]. [^{3}H]fNLLP, a synthetic analog of fMLP [see Ref. 16] was used at a final concentration of 1.1×10^{-8} M. Nonspecific binding was defined as the amount of [^{3}H]fNLLP bound by cells in the presence of 10^{-6} M unlabeled fNLLP, a value found to be 30–50% of the total binding. Data are mean values of triplicate samples.

Data analysis. Wherever appropriate, statistical analyses of the data were done using the *t*-test for independent samples, the paired *t*-test or the Wilcoxon signed rank test as described by Snedecor and Cochran [17]. In some experiments, IC_{50} values were obtained by visual inspection of the inhibition curves and are expressed as geometric means of three or more experiments plus or minus the standard error of the mean.

RESULTS

Migration. Rat PMN pretreated with 0.01 to 0.1 mM azapropazone for 10 min at 37° were inhibited in their ability to migrate in response to the chemotactic peptide fMLP (Fig. 1). In seven separate experiments, control, untreated PMN migrated 24.5 ± 1.1 microns into filters in response to fMLP (3.3 nM), whereas PMN pretreated with 0.1 mM azapropazone migrated only 18.4 ± 1.9 microns. This inhibition in stimulated migration relative to

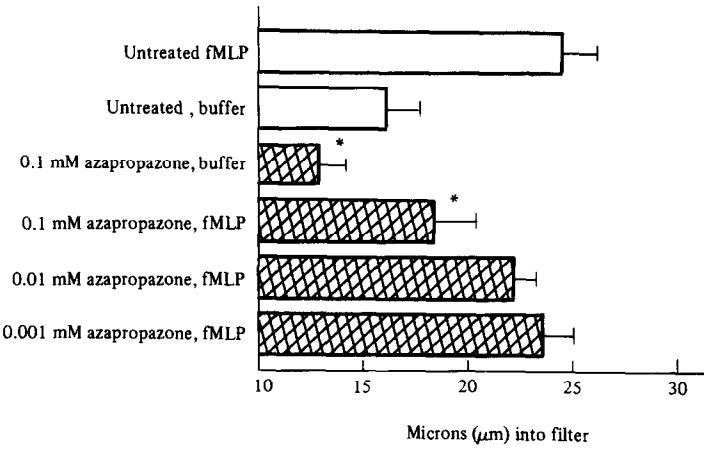

Fig. 1. Effects of azapropazone on rat neutrophil migration. Rat neutrophils were treated with or without azapropazone for 10 min at 37°. Cell migration was assayed in modified Boyden chambers using 3.0 μm pore size MF Millipore filters and 3.3 nM fMLP as a stimulus. Data are mean values of seven experiments and are expressed plus or minus the standard error. Statistical analysis was performed using a two-tailed *t*-test. (*) $P \leq 0.05$.

that of the untreated control cells was statistically significant at $P < 0.05$. In addition, PMN pretreated with 0.1 mM azapropazone were also inhibited significantly ($P < 0.05$) in their levels of unstimulated random migration (i.e. the response to buffer) when compared to the analogous, untreated control. Finally, PMN pretreated with 0.01 mM azapropazone showed slight (22.2 ± 1.7 microns), but statistically insignificant inhibition, and little, if any, inhibition in cell migration was seen with PMN treated with 0.001 mM azapropazone.

Aggregation. Azapropazone also caused a concentration-dependent inhibition of rat PMN aggregation in response to fMLP (Fig. 2). In six experiments, cells pretreated with 0.5 and 1.0 mM azapropazone were inhibited by 27 ± 7.5% and 34 ± 10.5% in their aggregation response to fMLP. In both instances, the degree of inhibition was statistically significant at $P < 0.05$. Concentrations of azapropazone less than 0.5 mM had no significant effect on the aggregation response.

Granule enzyme release. Azapropazone also inhibited β-glucuronidase release by rat PMN in response to fMLP (Fig. 3). In four experiments, PMN pretreated with 0.5 and 1.0 mM azapropazone were inhibited by 20–35% and 60–70% in the amount of β-glucuronidase released compared to untreated PMN. Again, this degree of inhibition seen at these two concentrations of azapropazone was statistically significant at $P \leq 0.05$. From these experiments, a mean IC_{50} value of 0.5 ± 0.1 mM ($N = 4$) was calculated.

Superoxide anion (O_2^-) production. Azapropazone also inhibited the O_2^- dependent reduction of cytochrome *c* by rat PMN stimulated with 100 ng/ml PMA. PMN pretreated with 0.1 mM azapropazone were inhibited significantly ($P < 0.05$) both in the rate of O_2^- generation and in the maximal amounts

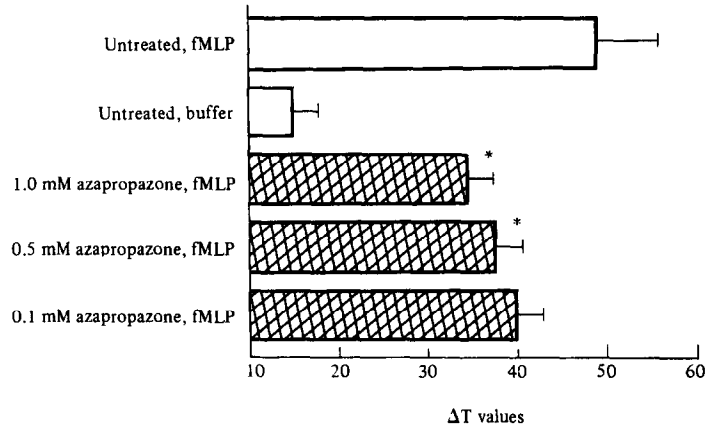

Fig. 2. Effects of azapropazone on rat neutrophil aggregation. Rat neutrophils were treated with or without azapropazone for 10 min at 37°. Aggregation was assayed in duplicate in a 4-channel model PAP-4 aggregometer, and 10^{-7} M fMLP was used as a stimulus. Data are mean values of six experiments plus or minus the standard error. Statistical analysis was done using a two-tailed *t*-test. (*) $P \leq 0.05$.

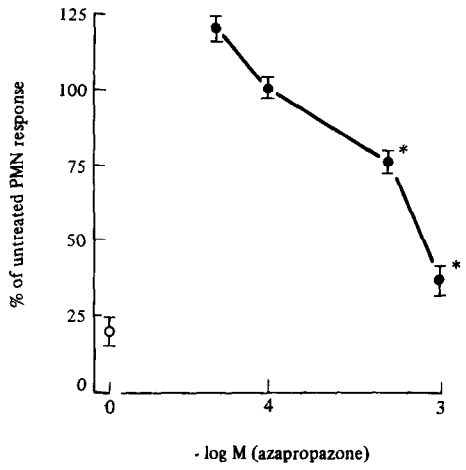

Fig. 3. Effects of azapropazone on rat neutrophil β-glucuronidase release. Rat neutrophils were treated with or without azapropazone for 10 min at 37°. Cytochalasin B (5 μg/ml) and fMLP (10^{-7} M) were added, and the mixture was incubated for an additional 10 min at 37°. The cells were pelleted at 4°, and aliquots of supernatant fluid were removed and assayed for β-glucuronidase activity. Data are mean values of four experiments plus or minus the standard error. Statistical analysis was done using a two-tailed *t*-test. The amount of β-glucuronidase released spontaneously by PMN is indicated (○). (*) $P \leq 0.05$.

of O_2^- generated within 15 min after PMA stimulation. Linear regression analysis of rat PMN O_2^- production between 0 and 15 min shows that control, untreated PMN produced O_2^- at a rate of 0.6 ± 0.1 nmole cytochrome *c*/min/5×10^6 PMN when stimulated with 100 ng/ml PMA (N = 6). In contrast, PMN pretreated with 0.1 mM azapropazone exhibited a 2- to 3-fold lower rate value of 0.25 ± 0.03 nmole cytochrome *c* reduced/min/5×10^6 cells, a difference statistically significant at $P < 0.05$. Rate values calculated for PMN pretreated with 0.01 mM azapropazone also showed a slight but statistically insignificant decrease (0.4 ± 0.1 nmole cytochrome *c* reduced/min/5×10^6 cells), and the value obtained with cells pretreated with 0.001 mM azapropazone (0.55 ± 0.1) was equivalent to the control value.

Similarly, cells pretreated with 0.1 mM azapropazone produced 20–60% less O_2^- than control untreated cells when stimulated with PMA and monitored for 30 min at 37° (Table 1). Again this difference was statistically significant at $P < 0.05$. Cells pretreated with 0.001 to 0.01 mM azapropazone showed some inhibition at earlier time points (0–10 min), but these values were not significantly different from the amounts of O_2^- produced by control cells. In summary, these data indicate that 0.1 mM azapropazone caused a significant inhibition in both the rate of O_2^- generation and the maximal amounts of O_2^- produced in PMN stimulated with 100 ng/ml PMA.

Binding studies. We next tested whether azapropazone inhibited these PMN functions by interfering with the binding of fMLP to the PMN membrane receptors for this peptide. Table 2 shows results obtained in one of two experiments. In this experiment, control, untreated PMN bound 16.6 ± 4.1 fmoles of [^{3}H]fNLLP, and PMN incubated with 0.01 to 1 mM azapropazone bound identical or greater amounts of the labeled peptide. Thus, the inhibitory effects of azapropazone on the PMN functional responses do not result from an ability of this drug to antagonize formylpeptide binding to rat PMN.

DISCUSSION

The data presented in this paper demonstrate that azapropazone inhibits a number of PMN functional responses which are important in the pathology of inflammatory disease and, in particular, gouty arthritis. In these studies, azapropazone was found to inhibit PMN migration, degranulation, aggregation and superoxide anion generation at concentrations of 0.1 to 1.0 mM. These concentrations are relatively close to the plasma levels of azapropazone achieved after oral administration of therapeutic or anti-inflammatory doses of the drug to both rats and humans. Using a peak plasma level of 160 μg/ml measured in rats by Jahn *et al.* [18] (see also review in Ref. 1), one calculates that rat blood levels of azapropazone reach 0.5 mM approximately 4 hr after dosing. Similarly, the therapeutic blood levels in humans are about 0.1 to 0.2 mM 4 hr after oral

Table 1. Inhibition of rat PMN O_2^- production by azapropazone

	(nmoles Cytochrome *c* reduced/5.0×10^6 PMN)					
Treatment	0 min	5 min	10 min	15 min	20 min	30 min
+ PMA	1.6 ± 1.3	5.4 ± 1.2	7.5 ± 1.6	7.8 ± 2.1	8.3 ± 2.4	8.1 ± 2.7
− PMA	0.3 ± 0.6	0.4 ± 0.4	0.5 ± 2.4	0.4 ± 0.8	0.1 ± 1.1	0.2 ± 1.8
PMA + 0.1 mM azapropazone	0.3 ± 0.6*	2.6 ± 1.4†	3.8 ± 1.6*	4.5 ± 2.0*	5.6 ± 2.4	4.8 ± 1.6
PMA + 0.01 mM azapropazone	0.3 ± 0.2*	4.7 ± 2.7	7.0 ± 3.8	8.3 ± 4.4	9.1 ± 4.4	9.7 ± 5.9
PMA + 0.001 mM azapropazone	0.2 ± 0.5*	4.9 ± 3.2	6.7 ± 4.4	8.7 ± 5.6	8.7 ± 5.6	8.7 ± 6.2

Rat PMN O_2^- production was measured as described in Methods using the methods of Babior *et al.* [11]. PMA (100 ng/ml) was used as the stimulus. Data are mean values obtained in five separate experiments and are expressed plus or minus the standard error of the mean. The 0-min time point is the Abs_{550} value immediately after PMA addition.

* Significantly different from untreated, PMA-stimulated PMN by paired *t*-test at $P < 0.05$.

† Significantly different from untreated, PMA-stimulated PMN by paired *t*-test at $P < 0.005$.

Table 2. Effects of azapropazone on [^{3}H]fNLLP binding to rat PMN

Azapropazone (mM)	[^{3}H]fNLLP bound (fmoles)	% of Control binding
None	16.6	100
1.0	22.5	135
0.1	17.2	103
0.01	24.8	149

Rat PMN (5×10^7 ml) were incubated with or without various concentrations of azapropazone for 10 min at 37°. Aliquots of the mixtures were then assayed for [^{3}H]fNLLP binding activity as described by Mackin *et al.* [16]. Data are mean values of triplicate samples and are corrected for nonspecific binding. Variation in the triplicate samples was less than 10% of the mean values.

administration of a 600 mg dose of azapropazone [19]. Both of these values approximate the drug concentrations we found effective at inhibiting several rat PMN functions *in vitro* such as cell migration, superoxide production, and degranulation. Taken together, these data and calculations suggest that the anti-inflammatory activity of azapropazone in treating gouty arthritis may be partly due to its ability to inhibit key PMN functions *in vivo*. Finally, these latter considerations also raise the question as to whether azapropazone therapy will have any significant effect on host defense since the drug inhibits several PMN functions important in this process. Obviously, this question warrants further investigation.

The mechanism by which azapropazone inhibits PMN functional responses remains unknown. Lewis *et al.* [20, 21] found that azapropazone inhibits enzyme release from rabbit liver and rat ileal lysosomes and suggested that this inhibition was due to lysosomal membrane stabilization by the drug. Such an effect could possibly explain the ability of azapropazone to inhibit PMN function since many of the responses assayed in our studies are at least partly dependent upon enzyme systems or mediators located in PMN lysosomes and which are released upon stimulation. In addition, azapropazone has been reported to modulate PMN metabolism specifically with regards to oxygen consumption and cellular respiration [22, 23]. Azapropazone as well as other NSAIDs (e.g. indomethacin) also block prostaglandin synthesis in macrophages and other cell types [24] but the relevance of these observations to our findings remains uncertain. Casein-elicited rat PMN as used in these studies synthesize little, if any, prostaglandins upon activation with several different stimuli (e.g. fMLP and A23187) ([25], W. Mackin, unpublished observation). Finally, other NSAIDs such as indomethacin [26] or phenylbutazone [27] have been reported to inhibit PMN responses to formylpeptides (e.g fMLP) by interfering with receptor binding. However, our findings that concentrations of azapropazone which maximally inhibit PMN biological responses do not decrease PMN [^{3}H]-fNLLP binding activity rules out this possibility. In addition, concentrations of azapropazone that inhibit PMN responses to fMLP also inhibit PMN O_2^- generation in response to the phorbol ester PMA, further suggesting that the inhibition of the PMN functions by azapropazone is not stimulus specific.

The ability of azapropazone to lower plasma urate concentrations and inhibit several key PMN functional responses makes it an attractive therapeutic agent for treating gouty arthritis. These combined pharmacologic activities also serve to distinguish azapropazone from other drugs currently used in treating gout. NSAIDs such as indomethacin and colchicine are used effectively to treat the disease but they have only anti-inflammatory activities and do not lower plasma urate concentrations. In addition, because both indomethacin and colchicine affect most PMN functional responses *in vitro* at concentrations 10- to 100-fold greater than the peak therapeutic plasma levels attained *in vivo*, their precise mechanisms of action in gout are uncertain [28, 29]. Inhibitors of purine metabolism such as allopurinol or uricosuric drugs such as probenecid also are used to treat gout because they cause a decrease in plasma urate levels [30]. However, neither allopurinol nor probenecid has any intrinsic anti-inflammatory activity and they often are used in combination with NSAIDs [30]. Based upon these considerations, the use of azapropazone for treating gouty arthritis may represent a distinct therapeutic improvement when compared to the current anti-gout drugs used clinically to treat the disease.

Acknowledgements—The authors would like to thank Kendra Marroni for her excellent secretarial assistance.

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