Primary Screening Approach for Anti-Inflammatory Agents Utilizing an *In Vivo* and a New *In Vitro* Method

R. A. CARRANO▲ and J. O. MALBICA

Abstract □ Several classes of standard pharmacological agents (approximately 38 different drugs) including anti-inflammatory, antiarthritic, analgesic, immunosuppressive, antihistaminic, autonomic, and CNS agents were tested in the *in vivo* carrageenin-inflamed rat paw test (Test I) and in an *in vitro* test involving their enhancement of the labilization of a purified rat liver lysosomal fraction by *N,N,N',N'*-tetramethylazoformamide (Test II). In general, there was good correlation between Test I and Test II results. The relationship of the findings to a screening program for anti-inflammatory agents is discussed.

Keyphrases □ Anti-inflammatory agents, screening program—correlation between *in vivo* carrageenin-induced edema and *in vitro* lysosomal-labilizing properties of *N,N,N',N'*-tetramethylazoformamide, rats □ *N,N,N',N'*-Tetramethylazoformamide lysosomal-labilizing properties—effect of anti-inflammatory agents, screening program, rats □ Screening programs, anti-inflammatory agents—correlation between *in vivo* carrageenin-induced edema and *in vitro* lysosomal-labilizing properties of *N,N,N',N'*-tetramethylazoformamide, rats

Laboratories differ in their programs for the screening of drugs for anti-inflammatory activity. Usually, a profile of tests progressing from those requiring less effort to the more difficult, time-consuming tests is used. The tests usually found at the front end of the program are suitable for rapid screening (*e.g.*, primary screening tests); others involve much greater effort but are often more selective (*e.g.*, secondary screening tests). It is not uncommon to find a drug active in some primary tests yet inactive in others. This undoubtedly stems from the different etiologies of the conditions generated in each test and the mechanisms involved in the drug's effect.

However, the main objective of the front end of the program should be to screen the compounds as selectively as possible (*e.g.*, keep the number of false-negative and false-positive conclusions to a minimum). In addition, such factors as cost and the scope of the screen are important. A program with a scope covering only antiarthritics would most likely be quite different from one designed to consider all anti-inflammatory agents or even analgesics.

In generating an effective *primary* screening program for anti-inflammatory agents, a question as to the use of *in vivo* or *in vitro* methodology arises. In considering the alternatives, two generally accepted primary screening models, an *in vivo* and an *in vitro*, were compared to estimate the incidence of false-positive or false-negative conclusions within several classes of pharmacological agents. It could then be estimated which of these methods, if either, would be the best to use on the front end of a screening program. Carrageenin-induced edema in the rat is used widely as an *in vivo* model for the primary screening of drugs for anti-inflammatory activity, and, more recently, the effect of drugs on lysosomal particles *in vitro* is being employed to screen rapidly for such drugs. The *in vitro* method utilized is a technique based on the finding by Malbica (1) that anti-inflammatory agents enhance the lysosomal-labilizing properties of *N,N,N',N'*-tetramethylazoformamide (I). This compound was shown by Kosower *et al.* (2) to oxidize glutathione stoichiometrically in red blood cells to the disulfide. In contrast, none of the nonsteroidal anti-inflammatory agents tested enhanced labilization induced by cysteine (3).

EXPERIMENTAL

***In Vitro* Screening**—*N,N,N',N'*-Tetramethylazoformamide was synthesized according to the method of Crawford and Raap (4). Rat liver mitochondrial–lysosomal fractions were prepared according to the method previously described by Malbica and Hart (5). The final mitochondrial–lysosomal suspension was in 0.25 *M* sucrose. All livers were obtained from SPF male rats weighing between 150 and 200 g. The rats were exsanguinated by decapitation prior to obtaining the livers.

The lysosomal enzymes chosen for assay were acid phosphatase, β-glucuronidase, aryl sulfatase, and *N*-acetyl-β-glucosaminidase.

Table I—Enhancement Effect of Anti-Inflammatory Agents on *N,N,N',N'*-Tetramethylazoformamide-Induced Lysosomal Labilization

	Percent Increase in Activity over Control (± *SE*)				
Agent at 10^{-4} *M*	Acid Phosphatase	β-Glucuronidase	Aryl Sulfatase	*N*-Acetyl-β-glucosaminidase	Mean Percent Increase in Activity
Aspirin	23 ± 4.5	11 ± 9.9[a]	22 ± 9.8[a]	50 ± 9.3	27
Chloroquine	18 ± 4.9	66 ± 7.8	0[a]	35 ± 11.5	30
Flufenamic acid	42 ± 6.7	60 ± 8.5	48 ± 5.9	55 ± 12.6	51
Hydrocortisone	37 ± 8.5	53 ± 5.7	48 ± 2.6	36 ± 8.8	44
Indomethacin	56 ± 5.8	55 ± 8.5	64 ± 17.6	86 ± 4.9	65
Mefenamic acid	59 ± 7.2	72 ± 5.7	41 ± 9.2	76 ± 8.2	62
Phenylbutazone	79 ± 13.8	24 ± 7.8	42 ± 7.8	55 ± 5.5	50

[a] Not significant at $p \leq 0.05$ (minimum of four animals).

Table II—Rating Scale to Be Used for Comparison between Drugs

Mean Percent Increase in Control N,N,N',N'-Tetramethylazoformamide-Induced Enzyme Activity	Rating
>70	>1.00
56–70	1.00
46–55	0.75
36–45	0.50
26–35	0.25
0–25	0.00

Acid phosphatase (E.C. 3.1.3.2) and β-glucuronidase (E.C. 3.2.1.31) activity determinations were made according to previously described methods (5). Aryl sulfatase activity (E.C. 3.1.6.2) was colorimetrically determined by using *p*-nitrocatechol sulfate as substrate. Incubation was at 37° for 1 hr. at pH 5.0 (sodium acetate buffer, 0.1 *M*). The reaction was stopped with four volumes of 0.2 *M* glycine buffer, pH 10.4, and the developed color was read at 515 nm. *N*-Acetyl-β-glucosaminidase (E.C. 3.2.1.29) activity was colorimetrically determined by using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as the substrate. Incubation was for 30 min. at 37° and pH 5.0 (sodium acetate buffer, 0.1 *M*), after which time the reaction was stopped by addition of four volumes of 0.1 *N* NaOH and the developed color was read at 400 nm. Lysosomal suspensions were assayed for nitrogen content by a micro-Kjeldahl technique and nesslerization.

For the screening method, the control incubation mixture contained the mitochondrial–lysosomal fraction suspension equivalent to 1.2–2.0 mg. of protein, sucrose–trihydroxymethylamine acetate buffer (0.25 *M* sucrose in 0.04 *M* buffer, pH 7.4), and N,N,N',N'-tetramethylazoformamide at 1×10^{-3} *M*. Test anti-inflammatory agents were added at the desired concentration. The final volume of the mixture was 4.0 ml., and incubation was for 60 min. at 37°. Water-insoluble test compounds were dissolved in absolute ethanol. The addition of ethanol solutions to the incubation mixture, where applicable, did not exceed a final concentration of 1% v/v of ethanol. This concentration of alcohol had no effect on the enzymatic activity of mitochondrial–lysosomal fractions. At the end of the incubation period, the entire mixture was centrifuged at $15{,}000 \times g$ for 15 min. at 0°. The resulting clear supernatant-containing free acid hydrolases (nonsedimentable activity) were then assayed.

The percentage that each test compound increased the release (as measured by an increase in activity) of the four individual enzymes in the presence of N,N,N',N'-tetramethylazoformamide was calculated according to the following formula:

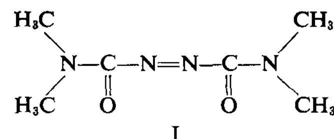
$$\frac{(x - y)}{y} \times 100 = \% \text{ increase in enzyme activity} \quad \text{(Eq. 1)}$$

where:

x = [activity (absorbance) of the enzyme in the presence of N,N,N',N'-tetramethylazoformamide and test compound]
y = [activity (absorbance) of the enzyme in the presence of N,N,N',N'-tetramethylazoformamide]

Statistical evaluations of the results were made using Student's *t* test.

***In Vivo* Screening**—Fasted (16-hr.) male albino rats (Wistar-derived, SPF) in the weight range of 160–180 g. were administered drugs by oral intubation (10 ml./kg.). Water was allowed *ad libitum*. All soluble drugs were dissolved in 0.9% saline; insoluble drugs were wetted with propylene glycol and suspended in 0.9% saline. Immediately following the oral administration, 0.1 ml. of a 1% sodium carrageenin[1] suspension in sterile saline was injected into the subplantar region of the right hind paw. The volume of the injected paw was then immediately measured by volume displacement and again at 4 hr. after drug administration. In general, six animals were used per test drug dose, and a control group receiving 10 ml./kg. of the drug vehicle was tested simultaneously for comparison.



The percent inhibition was calculated using the mean volume increase for each group, and a Student's *t* was determined to estimate the significance of each test.

RESULTS AND DISCUSSION

Table I illustrates how a number of reference anti-inflammatory agents performed in the *in vitro* test. All agents at 10^{-4} *M* enhanced the release of the lysosomal enzymes which was induced by N,N,N',N'-tetramethylazoformamide alone. Aspirin was the least active in this respect, while indomethacin produced the greatest increase. In general, the effect of an individual drug was similar for all of the enzymes. However, there were some cases (*e.g.*, the effect of chloroquine on aryl sulfatase and of phenylbutazone on β-glucuronidase), where there was a lesser or greater effect on one enzyme. In the case of phenylbutazone, it is known that this agent will directly inhibit the enzyme (6). Also, in the case of chloroquine, it was found that at 10^{-4} *M* there was an inhibition of the aryl sulfatase.

On the basis of these data, a rating scale based on the mean percent increase in enzyme activity was devised (Table II) to permit comparison between drugs. Thus, any compound showing a mean percent increase of less than 25% would be classified as inactive.

Table III reports how the various anti-inflammatory agents at 10^{-4} *M* appear when rated according to this scale.

Table IV compares the activity of drugs from different pharmacological classes in the two tests. A literature value for the dosage at which an accepted pharmacological effect of the drug can be seen is included in most cases to supply a dosage level comparison for the *in vivo* work. Several of the drugs listed under a category could very well be listed in more than one class but were placed only in one for simplicity.

In the anti-inflammatory–antiarthritic class, all compounds showed activity in both tests. In the *in vitro* test, the activity of aspirin and chloroquine was seen at 10^{-4} but not 10^{-5} *M*. The doses required for the various agents in the *in vivo* test were in general agreement with literature reports. The dose required for a significant effect with mefenamic acid was higher than is usually reported.

In the *in vivo* test, for a compound to be considered as a potential anti-inflammatory agent, it must show activity at a dose that does not produce other significant pharmacological effects (side effects). For example, amphetamine, listed in the autonomic class, will produce significant anti-inflammatory activity in the carrageenin test at 5 and 50 mg./kg. But at these doses, it produces marked autonomic, cardiovascular, and CNS effects and therefore cannot be considered as a potential anti-inflammatory agent. At more reasonable doses, for example, 2 mg./kg., where it still produces significant pharmacological effects, it does not produce significant anti-inflammatory activity. Thus, one must consider the doses required for accepted pharmacological responses when determining whether or not there is a false-positive result. In practice, when working with unknown drugs during the actual screening situation, the presence of other significant untoward pharmacology must be

Table III—Anti-Inflammatory Agents Rated According to Mean Enhancement Effect on N,N,N',N'-Tetramethylazoformamide-Induced Lysosomal Labilization

Agent	Rating (at 10^{-4} *M* Concentration)
Indomethacin	1.00
Mefenamic acid	1.00
Flufenamic acid	0.75
Phenylbutazone	0.75
Hydrocortisone	0.50
Aspirin	0.25
Chloroquine	0.25

[1] Marine Colloids, Inc.

Table IV—Activity of Several Classes of Pharmacological Agents in Two Types of Anti-Inflammatory Screening Tests

Drug	*In Vitro*	*In Vivo*			Dose, mg./kg. for Accepted Effect in Rats (*per os*) (Reference)
	Rating at 10^{-4} *M*	Dose, mg./kg. p.o.	Percent Inhibition of Edema		
Anti-Inflammatory, Antiarthritics					
Aspirin	0.25	100	18	S[a]	30–300 (10)
	0.00[c]	300	51	S	—
Chloroquine	0.25	30	0	NS[b]	>400
	0.00[c]	300	39	S	—
		500	45	S	—
Flufenamic acid	0.75	30	37	S	10–100 (11, 12)
	>1.0[c]	300	50	S	—
Hydrocortisone hydrochloride	0.50	30	44	S	6–18 (10)
	>1.0[c]	100	74	S	—
Indomethacin	1.00	1	9	NS	5 (13)
	1.00[c]	5	50	S	—
		10	55	S	—
Mefenamic acid	1.00	100	22	NS	10 (14)
		300	45	S	—
Phenylbutazone	0.75	30	38	S	30–90 (10)
	0.75[c]	100	45	S	—
Antihistamines and/or Antiserotonins					
Chlorpheniramine maleate	0.25	10	18	NS	0.1–12 (15)
	0.0[c]	30	41	S	—
		300 (2/6 deaths)	80	S	—
Cyproheptadine hydrochloride	>1.0	1	8	NS	0.08 (16)
	0.0[c]	10	0	NS	5 (17)
Pyribenzamine hydrochloride	0.0	10	0	NS	5 (17)
Immunosuppressives					
Cyclophosphamide	0.0	100	0	NS	5 (18)
6-Mercaptopurine	0.0	100	22	S	35 (19)
Methotrexate	0.0	5	21	NS	0.5 (19)
Analgesics					
Aminopyrine	0	100	45	S	200 (20)
		300	56	S	—
Codeine sulfate	0	50	17	NS	22.5 (16)
Meperidine hydrochloride	0	100	18	NS	100-200 (17)
		150	25	NS	—
Morphine sulfate	0	15	23	NS	15.4 (16)
		50	50	S	—
		100 (1/6 deaths)	39	S	—
Pentazocine lactate	0	100	9	NS	14 (21)
		200	4	NS	—
Phenacetin	0.75	100	24	NS	560 (17)
	0.50[c]	300	35	NS	—
		500	24	NS	—
Propoxyphene hydrochloride	0	75	8	NS	65
Salicylamide	0	100	0	NS	200 (17)
		500	0	NS	—
Autonomic Agents					
Amphetamine sulfate	0	2	0	NS	2 (16)
		5	40	S	—
		50 (1/6 deaths)	51	S	—
Atropine sulfate	0	50	28	NS	10 (16)
Dibenamine	1.0	30	10	NS	75–100[d] (22)
	1.0[c]	300	9	NS	—
Iproniazid phosphate	0	100	0	NS	100 (16)
		150	25	NS	—
Mecamylamine hydrochloride	0	10	24	NS	175[e] (23)
		50	68	S	2.5[f] (16)
Procaine hydrochloride	0	100	0	NS	200[g] (17)
Tolazoline	0	100	13	NS	1–2[h] (24)
		300	3	NS	—
Tranylcypromine sulfate	0	5	13	NS	5 (16)
		10[i]	38	S	—
CNS Agents					
Chlorpromazine hydrochloride	>1.0	10	0	NS	7 (16)
	0[c]	100[i]	65	S	—

Table IV—(*Continued*)

Drug	*In Vitro* Rating at 10^{-4} M	*In Vivo* Dose, mg./kg. p.o.	*In Vivo* Percent Inhibition of Edema	Dose, mg./kg. for Accepted Effect in Rats (*per os*) (Reference)
Imipramine	0.50	10	24 NS	20[g] (25)
	0[c]	50	0 NS	100[f] (16)
		100	39 S	—
Sodium phenobarbital	0	300[i]	28 NS	25 (14)
Trimeprazine tartrate	0.50	100	25 NS	—
	0[c]			
Miscellaneous				
Digitoxin	0	50	20 NS	94[e] (26)
2,4-Dinitrophenol	>1.0	30	11 NS	—
	0[c]			
Furosemide	0	30	0 NS	2820[e] (26)
		300	55 S	—
Papaverine hydrochloride	0	50	29 NS	—
Sodium chloride (0.9%)	0	10 ml./kg.	0 —	—

[a] S means significant at $p < 0.05$. [b] NS means not significant at $p > 0.05$. [c] Rating at 10^{-5} M. Incubation for aryl sulfatase was 60 min. [d] LD_{50}, mice, intraperitoneal. [e] Oral LD_{50}, rats. [f] Oral in mice. [g] Intraperitoneal. [h] Intravenous, dogs and cats. [i] Toxicity noted.

ruled out before a drug at a given dose level is considered as having true anti-inflammatory potential.

In the antihistamine and/or antiserotonin class, chlorpheniramine and cyproheptadine are both active at 10^{-4} M in the *in vitro* test. Cyproheptadine is a rather potent drug, as can be seen by its required oral dose for effect. Thus, when tested at a probably more physiological concentration, 10^{-5} M, it loses its activity in the *in vitro* test. In the *in vivo* test, all the drugs in this class would be considered as inactive or with no potential.

Neither test was able to pick out the immunosuppressive drugs. This fact is in favor of the selectivity of the methods but is disturbing if the intent of the screening program is to aim for all compounds that have antiarthritic potential, which would certainly include immunosuppressive agents.

In the analgesic class, phenacetin showed up as active in the *in vitro* test at 10^{-4} and 10^{-5} M. This activity is interesting in that the drug does possess both antipyretic and analgesic properties—two effects generally associated with anti-inflammatory effects. It did not display significant anti-inflammatory activity in the *in vivo* test.

Aminopyrine was active in the *in vivo* test, but this is not surprising because the compound is reported to have anti-inflammatory activity. It was inactive in the *in vitro* test. Unfortunately, its toxicity (7) prevents its use in therapeutics.

In the autonomic agent class, dibenamine was active at both 10^{-4} and 10^{-5} M in the *in vitro* test, whereas it was inactive in the *in vivo* test. This compound's high lipid solubility and capability of acting as an alkylating agent may enable it to have a direct effect on the lysosomal membranes (7, 8).

In the CNS class, chlorpromazine, imipramine, and trimeprazine were all active at 10^{-4} M in the *in vitro* test. Only imipramine was active at a reasonable dose level in the *in vivo* test. Chlorpromazine was shown (9) to disrupt lysosomes in cell culture. Trimeprazine, being a phenothiazine, probably acts similarly. At 10^{-5} M, the effect of both phenothiazines was no longer seen. Imipramine was not active at 10^{-5} M; but since it is not an extremely potent compound, it should not have shown activity at 10^{-4} M.

In the miscellaneous class, 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, was active at 10^{-4} but not 10^{-5} M in the *in vitro* test. This activity is unexplained. Furosemide was active at 300 mg./kg. but not 30 mg./kg. in the *in vivo* test.

In conclusion, false-positives occur with both of the tests discussed here, with only a little overlap between drugs. Therefore, it is suggested that the most selective way of screening for anti-inflammatory agents on the primary level would be to evaluate the compounds in both tests with the requirement that they show activity *in both* in order to be further considered. If this were the case here, the only drug out of 38 compounds tested that would have fooled us would have been imipramine.

On the other hand, it appears that neither test is able to predict immunosuppressive agents effectively, thus requiring other methodology for the primary screening of drugs that are potential immunosuppressives, including antiarthritics which could be acting by that mechanism. The rat adjuvant arthritis test would be suitable but, unfortunately, requires much more compound, effort, and time to complete.

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Complexation of Acetaminophen with Methyl Xanthines

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Abstract ☐ The interaction, in aqueous solution, of acetaminophen with caffeine, theophylline, and theobromine was investigated. Caffeine and theophylline form isolatable crystalline 1:1 complexes with acetaminophen, while theobromine apparently does not complex with acetaminophen. The caffeine–acetaminophen complex was found to exist in three forms, differing only in the degree of solvation. The solubility and dissolution rates of some of the caffeine–acetaminophen and theophylline–acetaminophen complexes were determined.

Keyphrases ☐ Acetaminophen—complexation with methyl xanthines, particularly caffeine and theophylline ☐ Caffeine–acetaminophen complexes—isolation, solubility, dissolution rates, stability constants ☐ Theophylline–acetaminophen complexes—isolation, solubility, dissolution rates, stability constants ☐ Complex formation—acetaminophen with methyl xanthines ☐ Analgesic availability—effect of complexation

Many studies have shown that caffeine and related xanthines are capable of interacting in aqueous media with a variety of drugs through complexation (1).

In a recent search of the literature relative to the possible influence of complexation on the availability of analgesics, it became obvious that the effect of the interaction of acetaminophen with the methyl xanthines had not been reported.

Although acetaminophen and caffeine are present in many commercial analgesic products (2), the role or purpose of caffeine as a therapeutically active ingredient may be doubtful. However, such combinations may exhibit physical–chemical interactions of pharmaceutical interest and importance from a formulation standpoint.

EXPERIMENTAL

Equipment[1]—Constant-temperature water baths were used which maintained the temperature at 25 ± 0.1°. Tablets for the dissolution studies were prepared using a Carver press and a flat-face punch and die with a diameter of 1.27 cm. (0.5 in.). The dissolution apparatus consisted of a 500-ml., two-necked round-bottom flask immersed in a constant-temperature bath. The dissolution medium (350 ml. of water) was stirred through the central neck of the flask using a stirring motor[2] and an L-shaped glass stirrer located 2.5 cm. above the bottom of the flask. The stirring rod was fashioned from a 7-mm. glass rod, as shown in Fig. 1.

Materials—Caffeine, theophylline, theobromine, and acetaminophen were recrystallized from distilled water, dried to constant weight, and shown to be anhydrous by differential scanning calorimetry and/or NMR. Melting points were found to be in good agreement with literature values (3). All water used was distilled from acid permanganate solution in an all-glass apparatus. The NMR spectra were obtained in deuterated dimethyl sulfoxide (97.5% isotopic purity). All other reagents were of analytical or reagent grade.

Methods—*Solubility Study*—A certain quantity of substrate, in excess of its aqueous solubility, was placed in 30-ml. glass, screw-capped vials together with increasing but accurately weighed amounts of ligand (Table I) and 20 ml. of distilled water. The vials were sealed and fixed on a rotating shaft in a constant-temperature bath and equilibrated for 48 hr. at 25 ± 0.1°. Aliquot portions of the

Table I—Amounts of Substrate and Ligand Used in the Various Solubility Studies

Substrate	Ligand
Acetaminophen, 2 g.	Caffeine, 0–3.1 g.
Acetaminophen, 1.5 g.	Theophylline, 0–2.4 g.
Theobromine, 0.03 g.	Acetaminophen, 0–0.32 g.

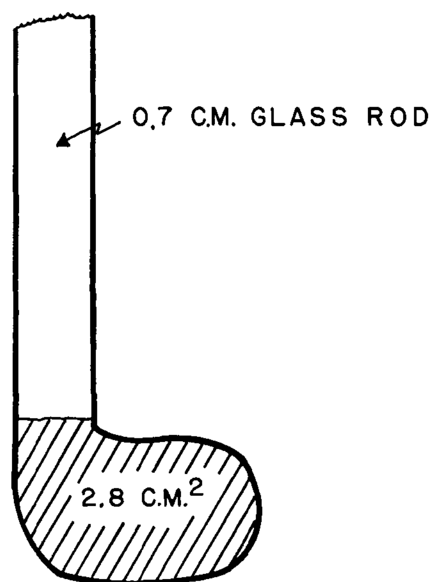

Figure 1—*Shape and dimensions of glass stirrer used in dissolution rate studies.*

[1] Spectrophotometric measurements were made using a Cary model 14 or 15 spectrophotometer. Differential scanning calorimetry was performed using a Perkin-Elmer model DSC-1B differential scanning calorimeter. The Varian model T-60 was used for obtaining NMR spectra.

[2] Inframo model RZR64.