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Evidence for the Existence of High Affinity Binding Sites for Indomethacin on Human Blood Platelets

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SUMMARY

Using human blood-washed platelets and [^{3}H]indomethacin, we demonstrated the presence of saturable, time- and temperature-dependent high affinity binding sites for non-steroidal anti-inflammatory drugs. The observed K_d value for indomethacin was 5 nM. Structural specificity of the non-steroidal anti-inflammatory drug site was studied with arylacetic acids, anthranilic acids, and compounds from other chemical families. Arylacetic acid drugs had affinities which were similar to the affinity of indomethacin. Affinity differences among the other drugs may be related to the presence or absence of the lipophilic substituent on the central ring. As expected, anti-inflammatory pyrrazole derivatives, aspirin, bucloxic acid, cortisol, nordihydroguaiaretic acid, and the chemotactic peptide formyl-Met-Leu-Phe were not recognized by the indomethacin binding site.

Blood platelets metabolize arachidonic acid by a reaction due to a membrane enzyme, cyclooxygenase (EC 1.14.99.1). Aspirin, but not salicylic acid, irreversibly inhibits the enzyme activity by direct acetylation of the catalytic site, while indomethacin acts as a noncompetitive inhibitor (1–3). Its inhibitory site is probably different from the substrate site. Another binding site regulating cyclooxygenase activity has also been postulated (4). Moreover, various NSAIDs which inhibit cyclooxygenase activity influence the inhibitory effect of aspirin (5, 6).

The therapeutic activity of NSAIDs is presumably due to their interaction with the cyclooxygenase enzyme, and the term of "receptor" has been suggested on the basis of the structural similarity of NSAIDs and their parallel inhibitory activity (7). It has been postulated that binding of NSAIDs to the cyclooxygenase receptor would prevent stimulation of cyclooxygenase activity by the chemical mediators of inflammation (8). However, such a binding site has never been identified directly.

In this paper, we demonstrate the presence of a high affinity binding site for indomethacin on washed human blood platelets and the specificity of this site for NSAIDs structurally related to indomethacin.

Materials and Methods

Reagents

[^{3}H]Indomethacin (10 Ci/mmol) was from Amersham (United Kingdom). BSA (fraction V powder) was from Fluka AG. The following NSAIDs were used: indomethacin and sulindac, Merck-Sharp-Dohme-Chibret; diclofenac, phenylbutazone, and oxyphenbutazone, CIBA-Geigy; ketoprofen and metiazinic acid, Specia; CHPPA and its reduced derivative (CHPPA-H2), Logeais; clomethacin and naproxen, Cassenne; mefenamic acid and flufenamic acid, Parke-Davis; ibuprofen, Dacour; benoxaprofen, Eli Lilly and Co.; bucloxic acid, Clin-Midy-Sanofi; nifluric acid, UPSA; and pipebuzone, Dausse.

Platelets

Human blood platelets from donors were prepared by centrifugation (400 × *g*, 30 min). Citrate-EDTA solution was used to prevent platelet adhesion during centrifugation. Platelet-rich plasma contained about 5×10^{10} platelets in 50 ml. Before the binding assays, cells were washed twice and resuspended in PBS, pH 7.5, containing 0.1% BSA (PBS-BSA) (final concentration, 20×10^6 platelets per ml). Viability of the cells was 90–95% as determined by trypan blue exclusion.

Binding Studies

Association. [^{3}H]Indomethacin (5 nM) was added to the platelet suspension and incubated at different temperatures (4, 20, 30, and 37°). At specified times, 0.2 ml of the suspension was removed, diluted with 0.6 ml cold PBS-BSA, and centrifuged in a Microfuge. The supernatant was discarded and 0.1 ml of 10% $HClO_4$ was added to the cell pellet. The pellet radioactivity was measured in 8 ml of ACS (Amersham) in a liquid scintillation counter.

Nonspecific binding was determined by incubating cells with the tracer in the presence of an excess (× 200) of unlabeled indomethacin. Specific binding was the difference between total binding (tracer alone) and nonspecific binding (tracer + 1 μM unlabeled indomethacin). In each experiment, incubations were carried out in triplicate and the variation among these replicates was 10% or less.

Dissociation. After 20 min incubation at 30° with tracer alone or tracer plus unlabeled indomethacin, the cells were centrifuged at 200 × *g* for 2 min. The supernatant was discarded and the cell pellet was resuspended in the same volume of ice-cold PBS-BSA. Then, 0.2-ml aliquots of the cell suspension were diluted in 2 ml of PBS-BSA (10-fold dilution) with or without 500 nM unlabeled indomethacin and incubated at 30°. At specified times, duplicate samples of 2-ml suspen-

ABBREVIATIONS: NSAIDs, non-steroidal inflammatory drugs; BSA, bovine serum albumin; CHPPA, (cyclohexyl-4′-phenyl-2)propionic acid; PBS, phosphate-buffered saline.

sion were centrifuged at 200 × g for 5 min. The radioactivity of the cell pellet was determined as previously described for the association studies.

Saturation analysis. Various amounts of [^{3}H]indomethacin (3 nM to 200 nM) were incubated for 20 min at 30° with (or without) 1 μM unlabeled indomethacin (final volume 0.2 ml). Specific binding was determined as for the association studies.

Competition studies. The inhibition of [^{3}H]indomethacin binding to platelets by several unlabeled NSAIDs was determined by incubating cells with [^{3}H]indomethacin (5 nM) for 20 min at 30° in the presence of various concentrations of these compounds. [^{3}H]Indomethacin specific binding was determined as for the association studies.

Results

Time course of binding. Specific binding of [^{3}H]indomethacin to platelets at 37° was maximal after 8 min incubation. Lowering the incubation temperature from 37° to 30°, 20°, or 4° decreased the specific binding at 10 min from 75 to 49, 22, and 8 fmol, respectively (Fig. 1). The following experiments were carried out at 30°.

[^{3}H]Indomethacin specific binding increased linearly with cell concentrations up to 40 × 10^6 cells/ml. Routinely, 20 × 10^6 cells/ml were used in the following studies.

Dissociation. When the association of [^{3}H]indomethacin to platelets reached a plateau, a 10-fold dilution of the incubation medium with buffer alone or buffer plus 500 nM unlabeled indomethacin permitted an assessment of the rate of dissociation of bound [^{3}H]indomethacin with a half-time of 40 min. This dissociation rate was not significantly modified by addition to the medium of excess unlabeled indomethacin (Fig. 2).

Saturation of binding sites. When increasing concentrations of [^{3}H]indomethacin (from 3 to 200 nM) were incubated with platelets for 20 min at 30°, the curve of specific bound radioactivity versus tracer concentrations (Fig. 3) reached a plateau. Nonspecific binding increased linearly with ligand concentration up to 200 nM. The Scatchard plot obtained was linear, suggesting the existence of one population of high affinity binding sites for [^{3}H]indomethacin (Fig. 3). A statistical analysis of dissociation constants (K_d) determined from the Scatchard analysis of six separate experiments with platelets prepared from different donors yielded a K_d of 6.1 ± 1.8 nM (mean ± standard deviation).

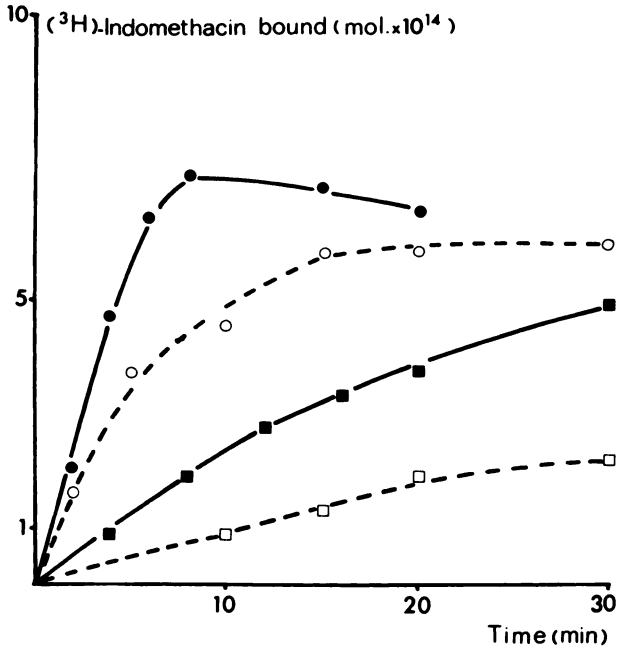

Fig. 1. Time course of [^{3}H]indomethacin binding to platelets. [^{3}H]Indomethacin (5 nM) was incubated with washed platelets (20 × 10^6/ml) in the absence (total binding) or the presence (nonspecific binding) of 1 μM unlabeled indomethacin. Bound and free radioligand was separated by centrifugation. Specific binding (difference between total and nonspecific binding) was plotted as a function of time. Each value is the mean of triplicate determinations. ●——●, 37°; ○——○, 30°; ■——■, 20°; □——□, 4°.

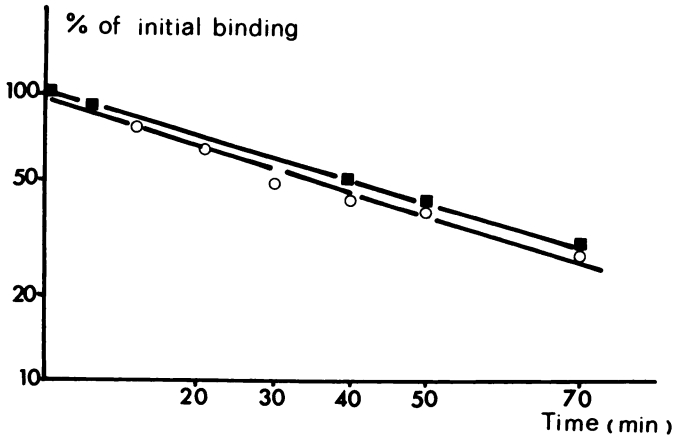

Fig. 2. Dissociation of [^{3}H]indomethacin binding to platelets. [^{3}H]Indomethacin remaining specifically associated with cells after a 10-fold dilution of the incubation medium was expressed as the percentage of ligand binding immediately after dilution at zero time (initial binding) and plotted as a function of time. ■——■, 10-fold dilution alone; ○——○, 10-fold dilution + 500 nM unlabeled indomethacin.

Competitive experiments with NSAIDs. [^{3}H]Indomethacin binding to human platelets was competitively inhibited by increasing concentrations of structurally related unlabeled NSAIDs (Fig. 4). The most potent inhibitors (from IC_{50} values listed in Table 1) were indomethacin, diclofenac, ketoprofen, and CHPPA. The curves of competitive binding were parallel for the different NSAIDs, except for benoxaprofen and aspirin (Fig. 4) which interacted differently with the binding sites.

Conformational structures of the different NSAIDs used in this study are illustrated in Fig. 5. Pluto views were carried out by J. P. DeClercq from the University of Louvain-la-Neuve (Belgium) and by Mrs. P. Briard from the University of Abidjan (Cote d'Ivoire). The Nourep view of indomethacin (Fig. 5A) was reported previously (9).

Discussion

On the basis of structure-activity relationships for indomethacin analogues, an anti-inflammatory receptor site has been postulated to consist of "a cationic center and two non-coplanar hydrophobic regions." This hypothesis was suggested by Shen and colleagues (7, 8) for indomethacin and by Sherrer (10) for *N*-arylanthranilic acid.

This paper provides evidence for the presence of one class of high affinity (nanomolar K_d range) binding sites for [^{3}H]indomethacin on human blood washed platelets. Any significant differences in the binding parameters of [^{3}H]indomethacin to platelets was observed when platelets were prepared from sev-

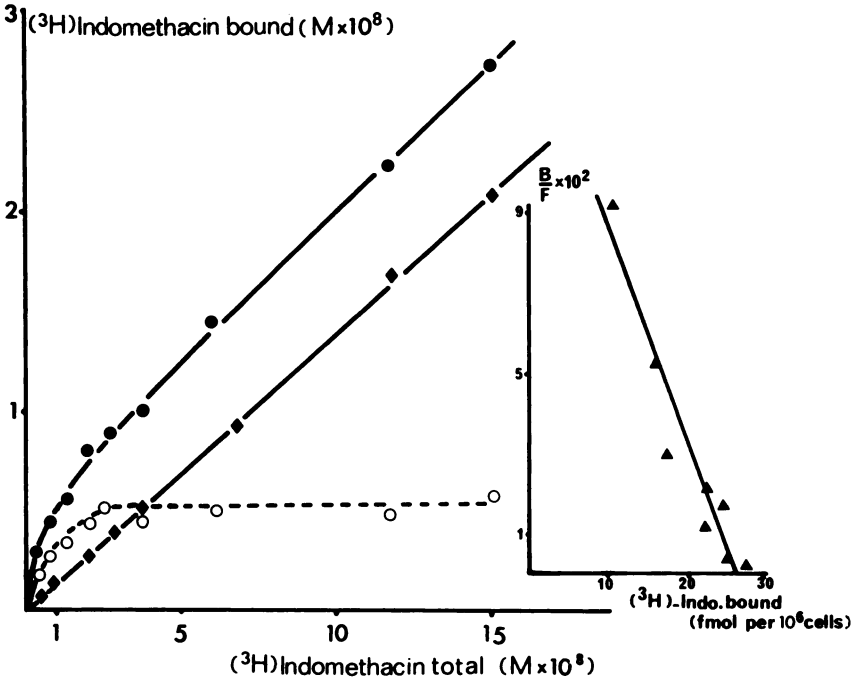

Fig. 3. Association of [^{3}H]indomethacin with platelets at steady state. Various concentrations of [^{3}H]indomethacin (3–200 nM) with (◆——◆) or without (●——●) unlabeled indomethacin (1 μM) were incubated with cells for 20 min at 30°; bound and free tracers were separated by centrifugation. Binding was expressed as bound labeled indomethacin as a function of total tracer concentrations (○—○). Specific binding is the difference between total (●——●) and nonspecific binding (◆——◆). Results are means of six separate experiments. The *inset* represents the Scatchard plot from these data.

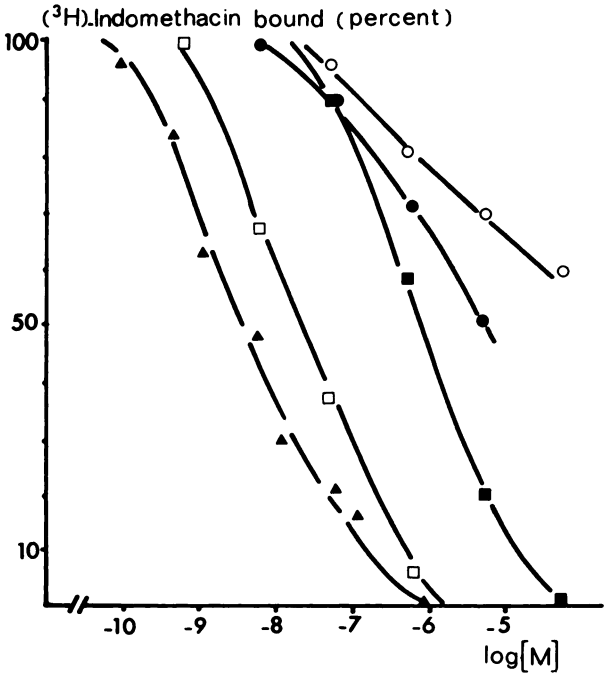

Fig. 4. Effects of typical NSAIDs on [^{3}H]indomethacin binding on human blood platelets. [^{3}H]Indomethacin (5 nM) was incubated with platelets for 20 min at 30° in the presence of varying concentrations of unlabeled NSAIDs. Results are expressed as the percentage of maximal specific binding (B/B_0 × 100) and plotted against concentrations of respective compounds. Results are means of three to six separate experiments. ▲, indomethacin; ●, benoxaprofen; ■, naproxen; □, ketoprofen; ○, aspirin.

TABLE 1
IC_{50} values from competitive studies with NSAIDs for [^{3}H]indomethacin binding to platelets

NSAID	IC_{50}
	nM
Indomethacin	5
CHPPA	8
Diclofenac	10
Ketoprofen	10
Mefenamic acid	50
Flufenamic acid	50
Niflumic acid	50
Clomethacin	80
Ibuprofen	300
Metiazinic acid	500
CHPPA-H2	500
Naproxen	750
Benoxaprofen	10,000
Sulindac	15,000
Bucloxic acid	60,000
Cortisol	>100,000
Pyrrazoles	>100,000
Aspirin	>100,000
FMLP	no interference
NDGA	no interference

eral donors. Association was a rapid process and dissociation demonstrated a $t_{1/2}$ = 40 min at 30°. Among the different NSAIDs tested, *N*-arylacetic acids and *N*-arylanthranilic acids were the most potent competitors for the [^{3}H]indomethacin binding site and were described as the most active drugs. Several anti-inflammatory and antalgic drugs such as pyrrazole derivatives (phenylbutazone, its metabolite oxyphenbutazone, and pipebuzone), as well as cortisol, possess little or no potency at the [^{3}H]indomethacin binding site, in agreement with their difference of structure with indomethacin. As expected, aspirin and nor-dihydroguaiaretic acid, an antioxidant and an inhibitor of H_2O_2 release (11), also did not affect [^{3}H]indomethacin binding to platelets. Moreover, it is of some interest to note that the chemotactic peptide f-Met-Leu-Phe did not interfere in this binding: indeed, Abita and colleagues (12) had shown that indomethacin was able to compete with ^{3}H-FMLP for binding to human neutrophils with an IC_{50} of 10 μM.

In order to bring out structural information on the ability of these NSAIDs to fit the same receptor, we have examined their

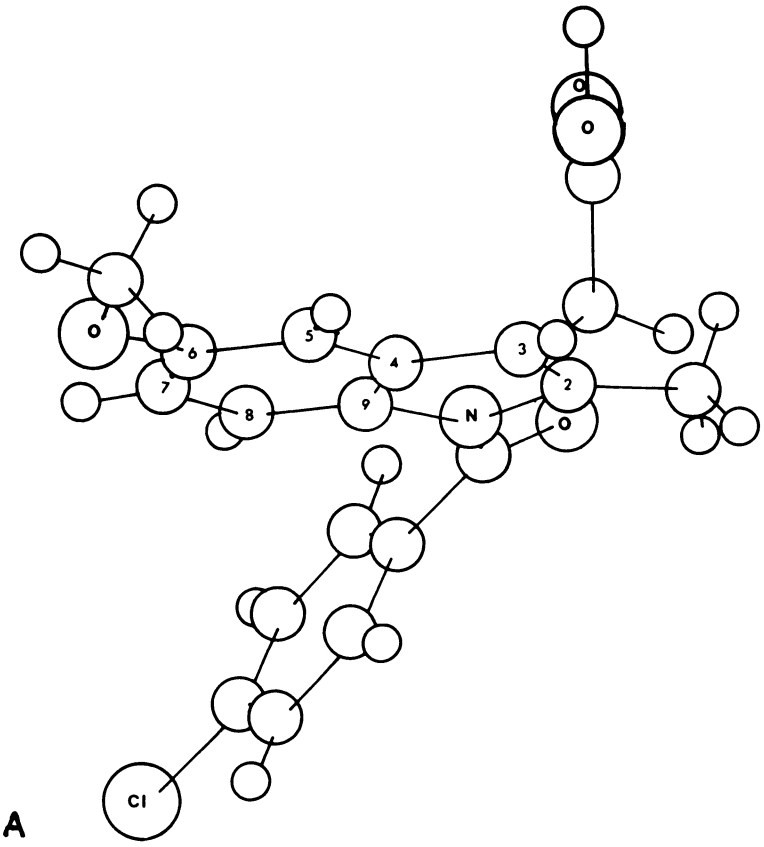

Fig. 5. Three-dimensional conformational structures of some NSAIDs showing the importance of the orientation of the carboxylic group and substituent for the anti-inflammatory activity. Indomethacin (*A*) was presented as a Nourep view and other NSAIDs (*B–H*) as Pluto views: *B*, clomethacin; *C*, ibuprofen; *D*, naproxen; *E*, diclofenac; *F*, sulindac; *G*, ketoprofen; and *H*, mefenamic acid.

three-dimensional molecular structure (Fig. 5) and found common spatial features. We must note that indomethacin bioactive conformation (as Nourep view, Fig. 5A) is essentially that observed in the crystal state (13).

Three main conditions seem to be necessary to get full biological activity (7, 8, 14): (a) a planar aromatic or heterocyclic system, (b) a carboxylic group outside the aromatic ring [called "carboxy-down conformation" following Shen's (8) formalism], and (c) a lipophilic substituant in the "anti" position with respect to the carboxylic group.

This spatial orientation is an important requirement for both anti-inflammatory activity and affinity for the binding site. The best example is the loss of affinity together with a decrease in observed anti-inflammatory activity with an indomethacin bioisoster, clomethacin (15, 16), in which only one conformer presents the bioconformation of indomethacin by pyramidal inversion of the nitrogen (Fig. 5B). In the crystal state, the *p*-chlorobenzoyl ring and the ester group are on the same side of the plane containing the indole ring ("syn"-conformation). The differences in affinity observed with the other potent *N*-arylacetic acid derivatives could be due to the shape of the substituent in "anti"-conformation with respect to the carboxylic head, such as ibuprofen (Fig. 5C), naproxen (Fig. 5D), mefenamic acid (Fig. 5H), CHPPA-H2, benoxaprofen, and sulindac (Fig. 5F). The importance of the lipophilic substituent is also evidenced by the existence of some potent new tricyclic NSAIDs (17–19).

Concerning the carboxylic group, we can conclude that an amplification of anti-inflammatory activity is generally observed when the acetic group is substituted by a methyl-acetic group: in these methyl-acetic NSAIDs, the most potent antipode presents an *S*-configuration as in naproxen (Fig. 5C), ketoprofen (Fig. 5G), ibuprofen (Fig. 5C) (20); introduction of a methyl group in the acetic acid moiety induces a preferred conformation with a carboxylic head oriented out of the aromatic ring (21). Moreover, on comparing anthranilic acids such as flufenamic acid, niflumic acid, and mefenamic acid (Fig. 5G) with diclofenac acid (Fig. 5D), one finds that derivatives incorporating a carboxyl group show a decrease for both anti-inflammatory activity and affinity: diclofenac acetic acid is reported to have 5 times the affinity of anthranilic carboxylic acids (Table 1). The lack of interference of bucloxic acid with the [^{3}H]indomethacin binding site is not suprising: this compound must be converted *in vivo* to an active metabolite with an acetic acid side chain to be effective as an anti-inflammatory drug (22).

These observations on the specificity of indomethacin binding sites on human blood washed platelets are in good agreement with the "NSAID-receptor" hypothesis of Shen and Sherrer (8, 10). However, no close correlation between binding and *in vivo* anti-inflammatory activity could be demonstrated, probably because of the large diversity of mechanisms involved in the inflammatory process. When we compared binding affinities on platelets for a variety of NSAIDs and their ability to inhibit cyclooxygenase activity on macrophages (23), a good correlation was observed ($r = 0.994$), in agreement with the hypothesis of the existence of an inhibitory site on the enzyme for this category of drugs. Direct radioligand binding experiments will undoubtedly be useful for assessment of structure-affinity relationship studies in the NSAIDs family.

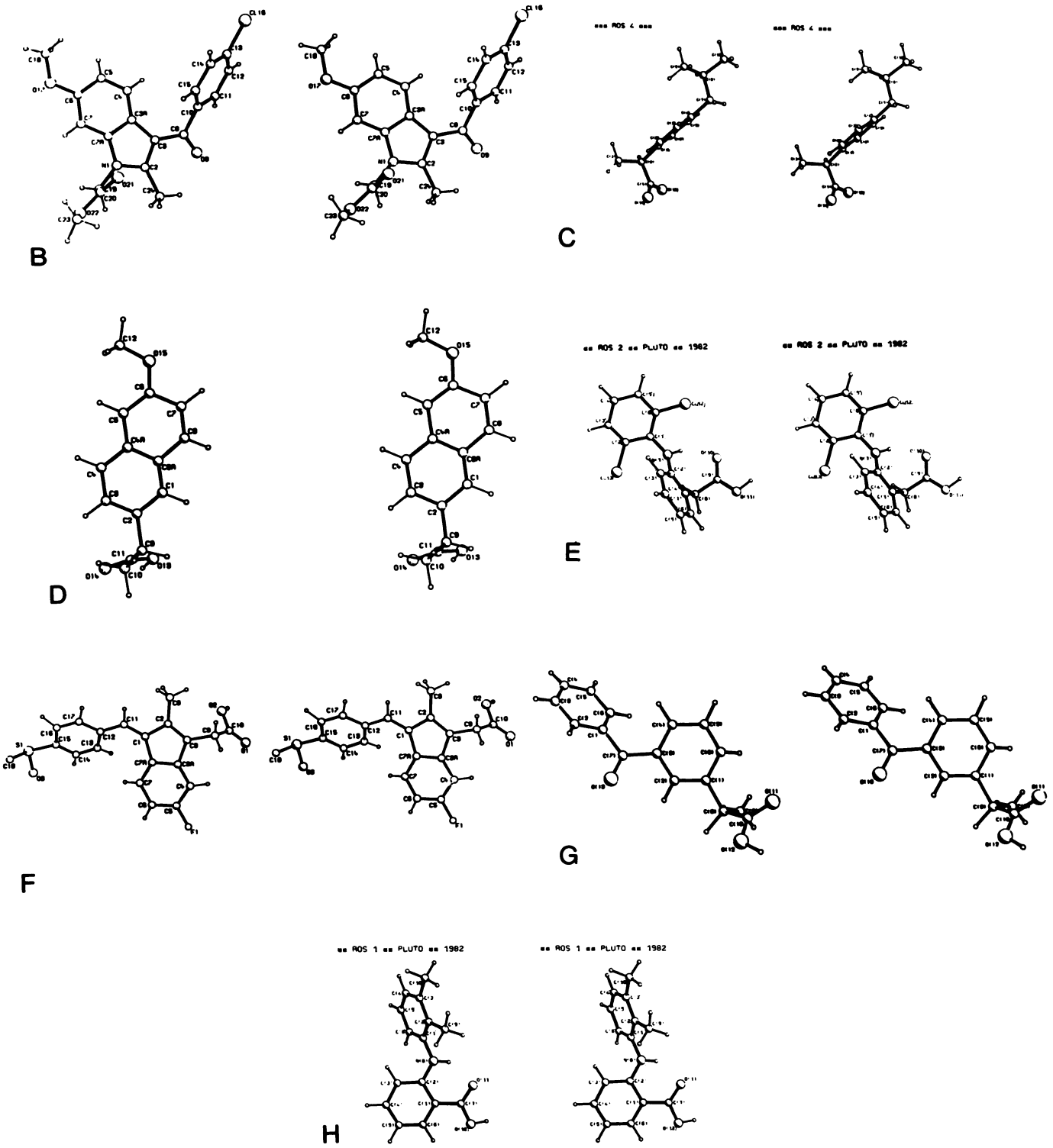

Fig. 5

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