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NON-STEROIDAL ANTI-INFLAMMATORY DRUG–COPPER COMPLEX MODULATION OF POLYMORPHONUCLEAR LEUKOCYTE MIGRATION

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Abstract—These studies were intended to compare the effects of aspirin, 3,5-diisopropysalicylic acid (3,5-DIPS), and indomethacin with those of their copper complexes: $Cu(II)_2(aspirinate)_4$, $Cu(II)_2(3,5\text{-}DIPS)_4$, and $Cu(II)_2(indomethacinate)_4$ as well as $Cu(II)_2(acetate)_4$ on polymorphonuclear leukocyte (PMNL) random and directional migration, in addition to their anti-inflammatory activities. Experiments were performed both *in vivo* and *in vitro*. *In vitro* modifications of PMNL migration were measured with the Boyden chamber using *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) as the chemoattractant and in the agarose assay using fMLP and serum chemotactic derivatives of complement as chemoattractants. *In vivo* anti-inflammatory activities of these compounds were determined after induction of a serum-induced pleurisy in the rat, and measurement of exudate volume and number of exudative cells 4 hr later. Copper complexes of non-steroidal anti-inflammatory drugs (NSAIDs) were found to be more effective in decreasing random migration and chemotaxis of PMNLs than their parent drugs or $Cu(II)_2(acetate)_4$ in *in vitro* studies. Only chemotaxis was found to be reduced significantly for PMNLs obtained from pleuritic rats after *in vivo* treatment and the order of copper complex effectiveness was: $Cu(II)_2(indomethacinate)_4 > Cu(II)_2(3,5\text{-}DIPS)_4 > Cu(II)_2(aspirinate)_4$. All doses of $Cu(II)_2(acetate)_4$ administered *in vivo* failed to affect chemotactic activity. Copper complexes of NSAIDs were also more effective than their parent drugs as anti-inflammatory agents, and $Cu(II)_2(acetate)_4$ had no anti-inflammatory activity in this model of inflammation. The order of anti-inflammatory activity was: $Cu(II)_2(indomethacinate)_4 > Cu(II)_2(3,5\text{-}DIPS)_4 > Cu(II)_2(aspirinate)_4$.

Concentrations of plasma copper complexes which are normal components of plasma increase 2- to 3-fold in a physiologic response to inflammation in humans and in animal models of inflammation [1–4]. Copper complexes of non-steroidal anti-inflammatory drugs (NSAIDs‡) have been found to be more effective than their parent drugs in many recognized models of inflammation [3, 5]. They are also less toxic than their parent drugs, they are not ulcerogenic, and they have potent antiulcer activity [3, 5–7]. In addition, copper complexes of NSAIDs are more effective analgesics than their parent drugs [8]. Since plasma copper complexes increase in response to inflammatory diseases, the above observations support the hypothesis that copper complexes of NSAIDs, formed *in vivo*, are active forms of these drugs and that the use of copper complexes in therapy of arthritic and other degenerative diseases [9] represents a physiological approach to treatment of these diseases.

Meacock *et al.* [10] compared the effects of D-penicillamine (0.68 mmol/mL of incubation medium), Cu(II)sulfate (nmol/mL), and the mixed valence copper complex of penicillamine $[Na_5Cu(I)_8Cu(II)_6(D\text{-penicillamine})_{12}Cl]$ (0.004 μmol/mL) on rat PMNL migration using the capillary tube technique. The mixed valence copper complex reduced migration by 83%, whereas Cu(II)sulfate and D-penicillamine only reduced migration by 25 and 16% respectively. These results obtained with the capillary method of determining effects on migration left open questions concerning effects of copper complexes on chemokinesis and chemotaxis as well as the influence of other copper complexes of NSAIDs on the PMNL response to inflammation. While it has been established that NSAIDs do inhibit chemotaxis, without affecting chemokinesis, of various pleuritic rat derived PMNLs [11, 12], the influence of copper complexes on these PMNLs has not been examined. Thus, it was of interest to compare the activity of copper complexes of NSAIDs on rat PMNL migration to that of their NSAID analogs and to investigate their activity on the evolution of an acute non-specific inflammatory reaction *in vivo*.

MATERIALS AND METHODS

Copper complexes of aspirin (Mallinckrodt

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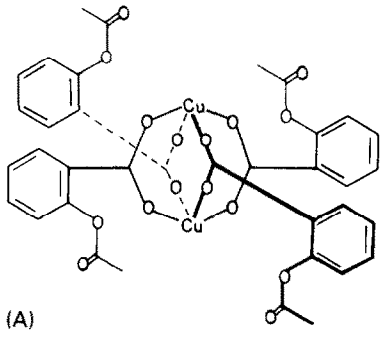
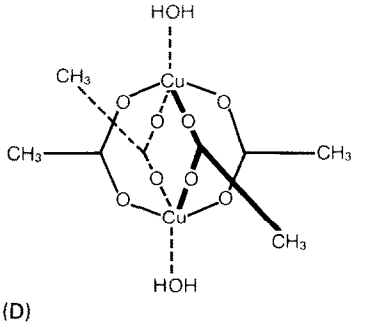
‡ Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; PMNLs, polymorphonuclear leukocytes; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; 3,5-DIPS, 3,5-diisopropylsalicylic acid; $Cu(II)_2(aspirinate)_4$, copper complex of aspirin; $Cu(II)_2(3,5\text{-}DIPS)_4$, copper complex of 3,5-DIPS; $Cu(II)_2(indomethacinate)_4$, copper complex of indomethacin; $Cu(II)_2(acetate)_4$, copper complex of acetic acid; $Cu(II)(salicylate)_2$, copper complex of salicylic acid; and $Na_5Cu(I)_8Cu(II)_6(D\text{-penicillamine})_{12}Cl$, mixed valence copper complex of D-penicillamine.



(A)



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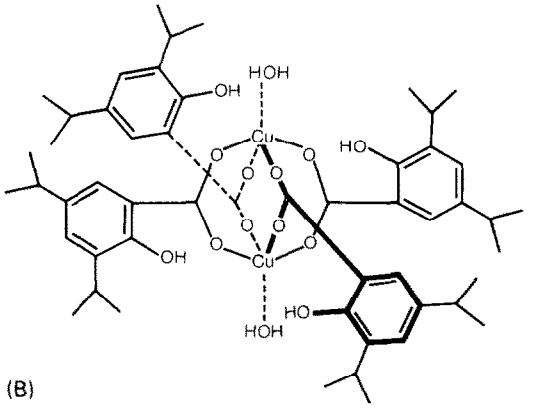
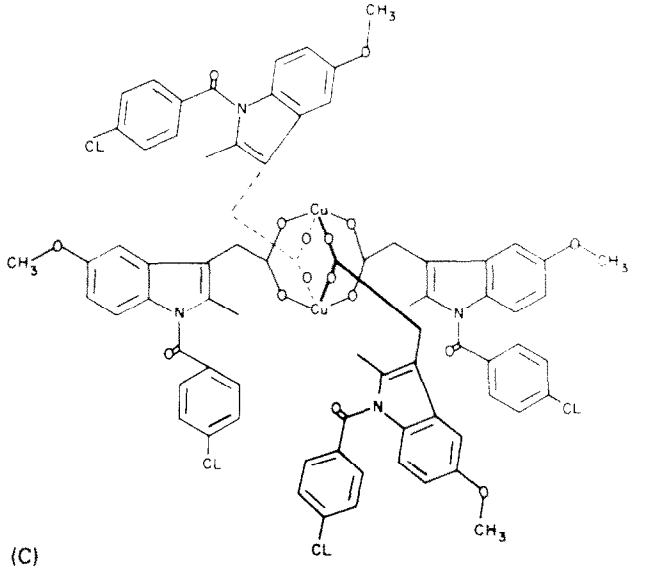


(B)

prepared and diluted appropriately for all *in vitro* experiments. Initially, 100 μL of the dimethyl sulfoxide solution was diluted with 5 mL of Hanks' Balanced Salt Solution. After vigorous stirring, the resultant suspension or solution was diluted with Hanks' Balanced Salt Solution to obtain the desired final concentration. PMNLs used in these experiments were collected from male Sprague–Dawley rats weighing 180–200 g (Depre Saint Doulchard, France) 4 hr after an intrapleural injection of 1 mL of isologous serum. Viability of these cells was accessed in all experiments by the trypan blue exclusion test.

For *in vivo* experiments, 100 μL (200 μL for aspirin) of a dimethyl sulfoxide solution of the test substance was suspended in an appropriate volume of 1% methylcellulose. After vigorous stirring, the intended dose was given by intragastric adminis-



(C)

Chemical Works, St Louis, MO), 3,5-diisopropylsalicylic acid (3,5-DIPS) (Aldrich Chemical Co., Milwaukee, WI), and indomethacin (Merck & Co., Rahway, NJ) were synthesized as previously described [5] without further purification of these ligands. All three complexes are binuclear: $Cu(II)_2(aspirinate)_4$ (A), $Cu(II)_2(3,5\text{-}DIPS)_4(H_2O)_2$ (B), and $Cu(II)_2(indomethacin)_4$ (C) [5, 13]. Cupric acetate dihydrate $[Cu(II)_2(acetate)_4(H_2O)_2]$ (D) was purchased from Mallinckrodt.

A dimethyl sulfoxide solution containing 100 times the highest concentration of the test substance was tration of 0.5 mL of this suspension 18 and 1 hr before the induction of the inflammatory reaction. Polymorphonuclear leukocytes were collected from the pleural cavity of these treated rats 4 hr after the injection of 1 mL of isologous serum into the pleural cavity. This model of pleurisy was used to evaluate the effectiveness of NSAIDs and their copper complexes in reducing the volume of exudate and the number of leukocytes in the exudate.

Chemotaxis (directional migration) and chemokinesis (non-directional migration) were determined using the Boyden [14] chamber technique as modified

by Keller *et al.* [15]. Agarose assay [16] experiments were performed in order to compare these results with those obtained with the Boyden chamber since different results have been described using these two techniques [17].

For the modified Boyden chamber technique, 5×10^6 cells suspended in 0.1 mL of Hanks' Balanced Salt Solution containing 1% bovine serum albumin (Sigma Chemical Co., St Louis, MO) were placed in the upper compartment, and 0.2 ml of chemoattractant, 10^{-8} M *N*-formyl-methionyl-leucyl-phenylalanine ((fMLP) (Sigma Chemical Co.) or isologous serum, was placed in the lower compartment. One 3 μM pore diameter cellulose filter (Millipore Corp., Strasbourg, France), was placed between these two compartments, and the chambers were incubated for 90 min at 37°. Migration was stopped by the addition of ethanol, and filters were stained with hemalum. Cell migration in five fields was assessed at high power magnification in triplicate using the leading front technique [18]. Mean and SEM observed distances (μm) were calculated, and paired samples were compared using Student's *t*-test.

For the agarose assay, 4 mL of 0.75% agarose (Indubiose A-37, Industrie Biologique Francaise, Villeneuve La Garenne, France) in Hanks' Balanced Salt Solution, pH 6.8, containing 10% heat-inactivated fetal calf serum was poured into small Petri dishes. Four sets of three wells were cut using a standard template. A suspension (5 μL) of 5×10^5 cells/mL was placed in the middle well, chemoattractant (5 μL) was placed in the outer well, and medium (5 μL) was placed in the inner control well. These dishes were incubated at 37° for 150 min, and directed migration, the chemotactic response, was measured as the distance (μm) traveled by cells from the border of the middle well toward the chemoattractant or non-directed random migration, the chemokinetic response, was measured as the distance (μm) travelled by cells toward the control well. Mean and SEM observed distances for these experiments done in quadruplicate were used to calculate the percent decrease in migration values and paired samples statistically compared using the ANOVA Fisher *t*-test.

RESULTS

Copper(II)$_2$(aspirinate)$_4$, Cu(II)$_2$(3,5-DIPS)$_4$, and Cu(II)$_2$(indomethacinate)$_4$ were more effective in reducing PMNL chemokinesis and chemotaxis than their parent ligands or Cu(II)$_2$(acetate)$_4$. As shown in Fig. 1, only the largest concentration of aspirin, 1500 g/mL (8326 nmol/mL), produced an inhibition of both chemokinesis and chemotaxis, while the lowest concentration of Cu(II)$_2$(aspirinate)$_4$, 150 μg/mL (177 nmol/mL), was effective in decreasing both of these parameters. In comparing aspirin and Cu(II)$_2$(aspirinate)$_4$, differences in effectiveness are made clearer by comparing molar concentrations of these two compounds, which is a comparison based upon number of molecules in each incubation. Data presented in Fig. 2 show that Cu(II)$_2$(3,5-DIPS)$_4$ was also more effective than 3,5-DIPS in reducing both chemokinesis and chemotaxis. These two figures show results observed using the

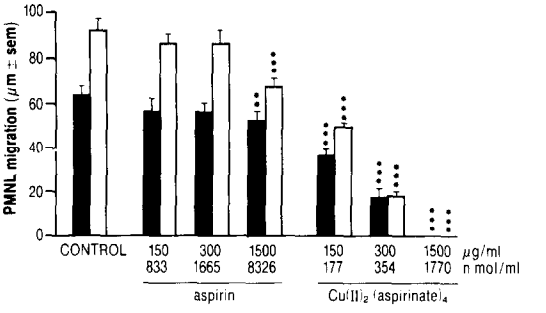


Fig. 1. Comparison of Boyden chamber non-directional (■) and directional (□) migration toward 10^{-8} M fMLP of normal rat PMNLs incubated with various concentrations of aspirin or Cu(II)$_2$(aspirinate)$_4$. Differences from the control value: ** $P < 0.01$ and *** $P < 0.001$ by ANOVA Fisher test, N = 6.

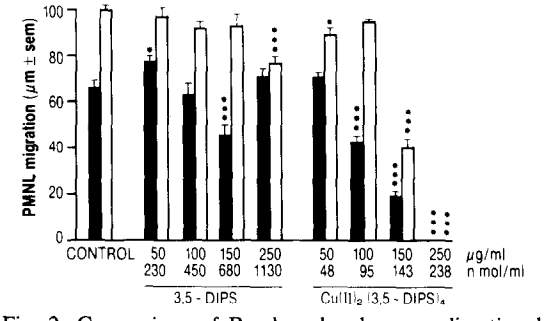


Fig. 2. Comparison of Boyden chamber non-directional (■) and directional (□) migration toward 10^{-8} M fMLP of normal rat PMNLs incubated with various concentrations of 3,5-DIPS or Cu(II)$_2$(3,5-DIPS)$_4$. Differences from the control value: * $P < 0.05$ and *** $P < 0.001$ by ANOVA Fisher test, N = 6.

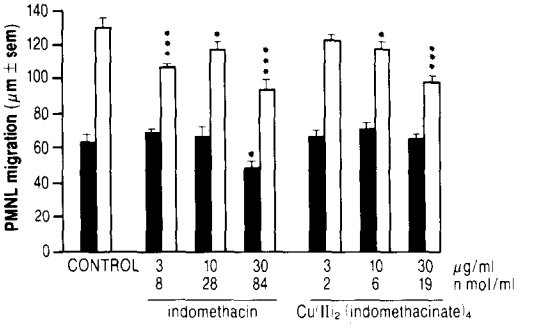


Fig. 3. Comparison of Boyden chamber non-directional (■) and directional (□) migration toward 10^{-8} M fMLP following incubation with various concentrations of indomethacin or Cu(II)$_2$(indomethacinate)$_4$. Differences from control value: * $P < 0.05$ and *** $P < 0.001$ by ANOVA test, N = 6.

Boyden chamber technique, but similar results were obtained using the agarose assay. In the comparison of indomethacin and Cu(II)$_2$(indomethacinate)$_4$ it was found using the Boyden chamber technique that they were both effective in reducing chemotaxis and that only indomethacin appeared to be effective in reducing chemokinesis at the highest concentration studied, 84 nmol/mL (Fig. 3). Similar results were obtained using the agarose assay. Concentrations higher than 50 μg/mL (150 nmol/mL) Cu(II)$_2$(acetate)$_4$ produced reductions of both chemotaxis and

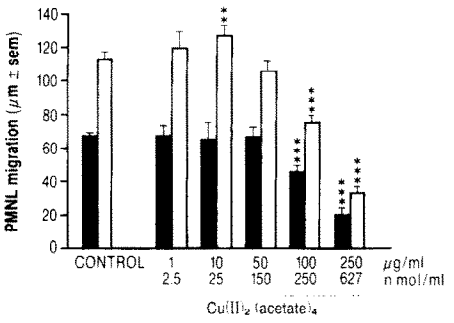


Fig. 4. Comparison of Boyden chamber non-directional (■) and directional (□) migration of normal rat PMNLs toward 10^{-8} M fMLP following incubation with various concentrations of $Cu(II)_2(acetate)_4$. Differences from control value: ** $P < 0.01$ and *** $P < 0.001$ by ANOVA Fisher test, N = 6.

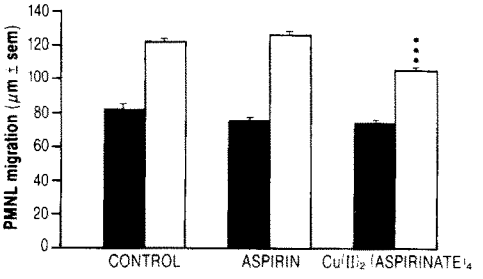


Fig. 5. Comparison of Boyden chamber non-directional (■) and directional (□) migration toward 10^{-8} M fMLP isolated from pleuristic rats treated with 500 mg/kg aspirin (2.78 mmol/kg) or $Cu(II)_2(aspirinate)_4$ (0.59 mmol/kg). *** Differences from the control value: $P < 0.001$, by Student's *t*-test, N = 6.

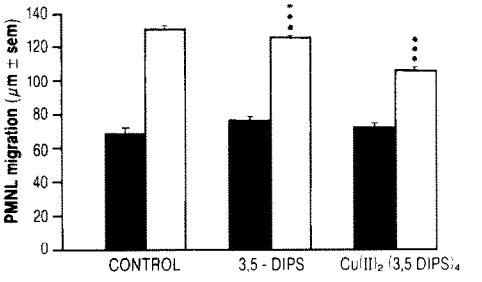


Fig. 6. Comparison of Boyden chamber non-directional (■) and directional (□) migration toward 10^{-8} M fMLP of PMNLs isolated from pleuritic rats treated with 250 mg/kg 3,5-DIPS (1.13 mmol/kg) or $Cu(II)_2(3,5\text{-DIPS})_4$ (0.24 mmol/kg). *** Differences from control value: $P < 0.001$, by Student's *t*-test, N = 6.

random migration (Fig. 4). However, these concentrations of $Cu(II)_2(acetate)_4$ greatly exceed concentrations of $Cu(II)_2(aspirinate)_4$, $Cu(II)_2(3,5\text{-DIPS})_4$, or $Cu(II)_2(indomethacinate)_4$ required to achieve reductions in chemokinesis and/or chemotaxis.

These three copper complexes were also found to be orally effective in treating isologous serum pleurisy, and they were more effective than their parent ligands. Data in Table 1 show that these complexes were four to five times as effective as their parent ligands, when numbers of molecules/dose are compared, in reducing inflammation, volume of pleural exudate, and number of leukocytes in the exudate. $Cu(II)_2(acetate)_4$ was ineffective in reducing pleural exudate and number of leukocytes at all doses studied.

Consistent with the anti-inflammatory results in this model of pleurisy, these three copper complexes did influence chemotaxis, whereas $Cu(II)_2(acetate)_4$ was again ineffective at all doses studied. A dose of 500 mg (0.6 mmol) of $Cu(II)_2(aspirinate)_4$/kg produced a reduction in chemotaxis while the same dose of aspirin (500 mg/kg, 3 mmol) was ineffective (Fig. 5). Copper$(II)_2(3,5\text{-DIPS})_4$ and $Cu(II)_2(indomethacinate)_4$ produced only slightly greater reductions in chemotaxis than 3,5-DIPS and indomethacin in these *in vivo* experiments (Figs 6 and 7). All doses of $Cu(II)_2(acetate)_4$ studied [1 (3), 3 (8), or 9 (24) mg/kg (nmol/kg)] failed to affect chemotaxis even though all three doses exceeded the dose of $Cu(II)_2(indomethacinate)_4$ found to be effective in reducing chemotaxis and provided larger amounts of

Table 1. Anti-inflammatory effects of NSAIDs and their copper complexes in the calcium pyrophosphate model of pleurisy

Compound	Dose mg/kg (mmol/kg)	Pleural exudate volume (ml)	Number of leukocytes ($\times 10^6$)
Vehicle	0	0.60 ± 0.15*	45 ± 10*
Aspirin	500 (2.78)	0.45 ± 0.12†	23 ± 8‡
$Cu(II)_2(aspirinate)_4$	500 (0.59)	0.40 ± 0.09‡	24 ± 7‡
Vehicle	0	0.90 ± 0.09	110 ± 14
3,5-DIPS	250 (1.13)	0.50 ± 0.11‡	34 ± 8§
$Cu(II)_2(3,5\text{-DIPS})_4$	250 (0.24)	0.50 ± 0.12§	32 ± 10§
Vehicle	0	0.80 ± 0.13	66 ± 12
Indomethacin	3 (0.008)	0.50 ± 0.10‡	31 ± 8‡
$Cu(II)_2(indomethacinate)_2$	3 (0.002)	0.60 ± 0.12‡	38 ± 9‡
Vehicle	0	0.60 ± 0.16	29 ± 10
$Cu(II)_2(acetate)_4$	1 (0.003)	0.65 ± 0.18	31 ± 11
	3 (0.008)	0.55 ± 0.15	41 ± 12
	9 (0.023)	0.80 ± 0.17	47 ± 10

* Mean of six values ± SD obtained in two experiments with N = 3.
†–§ Differences from vehicle-treated rats: † $P < 0.05$, ‡ $P < 0.01$, and § $P < 0.001$.

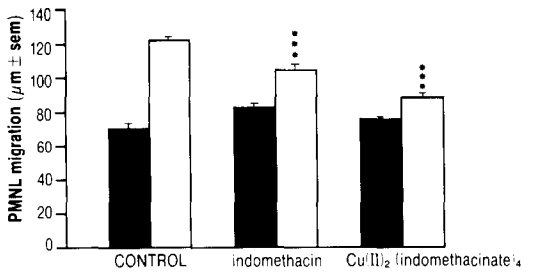


Fig. 7. Comparison of Boyden chamber non-directional (■) and directional (□) migration toward 10^{-8} M fMLP isolated from pleuritic rats treated with 3 mg/kg indomethacin (0.008 mmol/kg) or $Cu(II)_2$(indomethacinate)$_4$ (0.002 mmol/kg). *** Differences from the control value: $P < 0.001$, by Student's *t*-test, N = 6.

copper than provided by the dose of $Cu(II)_2$(indomethacinate)$_4$ used in these studies.

DISCUSSION

Since these ligands and their copper complexes vary greatly in molecular weight, the only valid comparison of their activities must be based upon molar concentration. With this as a basis for comparison it was always clear that these copper complexes were more effective than their parent ligands or $Cu(II)_2$(acetate)$_4$ in reducing chemotaxis by both the Boyden chamber and agarose methods of assay.

These copper complexes were also more effective anti-inflammatory agents than their parent ligands or $Cu(II)_2$(acetate)$_4$. The order of oral *in vivo* anti-inflammatory activity for these complexes was: $Cu(II)_2$(indomethacinate)$_4$ > $Cu(II)_2$(3,5-DIPS)$_4$ > $Cu(II)_2$(aspirinate)$_4$ based upon dose required to reduce the volume of exudate and number of PMNLs in the exudate. $Cu(II)_2$(acetate)$_4$ was either ineffective or exacerbated pleuritic inflammation. These orally administered complexes produced essentially the same order of reduction in pleuritic rat derived PMNL chemotaxis *in vivo* as their parent ligands with the exception of aspirin which produced no reduction in chemotaxis as has been reported previously [12].

The smaller number of molecules in a dose-weight of a copper complex compared to the same weight of parent ligand is due to the fact that molecular weights of these complexes are between four and five times greater than their ligand molecular weights. However, the amount of ligand in each complex is close to the amount of ligand in the corresponding dose of the parent ligand with the exception of the 15, 13 or 8% reduction in ligand weight due to the presence of copper in $Cu(II)_2$(aspirinate)$_4$, $Cu(II)_2$(3,5-DIPS)$_4$, or $Cu(II)_2$(indomethacinate)$_4$ respectively. These amounts of reduced ligand content of the administered dose may seem to be trivial but the reduction in ligand content would not favor equipotentency if potency were due to ligand content. Alternatively, it is suggested that the larger ligand dose is required to produce sufficient copper complex *in vivo* to allow the observed pharmacological effects. The source of copper required for the formation of these complexes *in vivo* is uncertain; however, plasma, liver, kidney, or bile copper are likely candidates [3]. The abundance of evidence showing that copper complexes of inactive ligands are active anti-inflammatory agents and that copper complexes of NSAIDs are more effective than their parent drugs [3, 5] also supports the suggestion that data presented here show that copper complexes of aspirin, 3,5-DIPS, and indomethacin are more effective anti-inflammatory agents and that this anti-inflammatory activity is at least partially accounted for by modulation of PMNL chemotaxis.

It is remarkable that very large concentrations of a dissociable form of copper, represented by $Cu(II)_2$(acetate)$_4$ which contains 32% copper, providing many times the normal total plasma concentration, 1 μg/ml, produced only weak inhibitions of chemokinesis and chemotaxis in these *in vitro* studies and produced no effect in these *in vivo* studies. There is no question that all of this copper was complexed following ligand exchange *in vivo* since the calculated concentration of ionic copper in plasma (10^{-18} M [19]), for example, is too small to be measured with existing equipment and amounts of copper in these doses do not exceed the complexing capacity of plasma. Complexes formed *in vivo* with this copper apparently do not have the required physicochemical properties needed to produce the desired pharmacological effects. NSAIDs must represent a class of ligands capable of forming complexes possessing the required physicochemical properties. In this regard, it is of special interest that $Cu(II)_2$(3,5-DIPS)$_4$, $Cu(II)_2$(indomethacinate)$_4$ and the $Cu(II)_2$(aspirinate)$_4$ dimethyl sulfoxide solvates are ether soluble and represent lipophilic compounds capable of transporting copper into lipid compartments. The order of lipid solubility: $Cu(II)_2$(indomethacinate)$_4$ = $Cu(II)_2$(3,5-DIPS)$_4$ ≫ $Cu(II)_2$(aspirinate)$_4$ may contribute to the propitious tissue, cellular, and subcellular distribution of these complexes which may account for the relative order of potency of these compounds as anti-inflammatory and antichemotactic agents.

It is tenable that increased activity of copper complexes is due to complexation which merely facilitates ligand absorption from the gut or passage through cell membranes since copper complexes are much more lipid soluble than the polar parent ligands. However, zinc complexes which are also more lipid soluble than their parent ligands have no anti-inflammatory activity while their copper complexes are active [5].

From results of the *in vitro* studies it is generally apparent that copper complexes were more effective in reducing chemotaxis than random migration. This is consistent with the observation that only the chemotactic activity of PMNLs was affected by treatment of pleuritic rats. The observed reduction in chemotaxis is remarkable since doses used in these studies were relatively small: 118 μmol/rat for $Cu(II)_2$(aspirinate)$_4$, 48 μmol/rat for $Cu(II)_2$(3,5-DIPS)$_4$, and 0.4 μmol/rat for $Cu(II)_2$(indomethacinate)$_4$. It is likely that these doses do not provide the extracellular concentration of complex necessary to cause a reduction in PMNL chemokinesis *in vivo*; however, they do provide enough complex, possibly nanomolar concentrations at the site of inflammation, to cause a reduction in chemotaxis.

The variation in anti-inflammatory dose of these copper complexes, 118 μmol/rat for $Cu(II)_2$(aspirinate)$_4$ to 0.4 μmol/rat for $Cu(II)_2$(indomethacinate)$_4$, is also interesting. This nearly 300-fold difference must be related to the variation in physicochemical properties of these complexes and their relative effectiveness in treating pleurisy.

Copper complexes of NSAIDs are less toxic than their parent drugs [3, 7, 20]. It is also most interesting that the principal chronic toxicity of NSAIDs, gastric irritation, is not observed with their copper complexes and these complexes are potent antiulcer agents [3, 7, 20]. Thus, the use of copper complexes may better be viewed as beneficial in therapies of inflammatory diseases [21].

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