Anti-Inflammatory, Antiproliferative, and Radical-Scavenging Activities of Tolfenamic Acid and Its Metal Complexes

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Some new complexes of tolfenamic acid (=2-[(2-methyl-3-chlorophenyl)amino]benzoic acid; Htolf) with potentially interesting biological activities are described. The complexes $[Mn(tolf)_2(H_2O)_2]$, $[Co(tolf)_2(H_2O)_2]$, $[Ni(tolf_2(H_2O)_2]$, $[Cu(tolf)_2(H_2O)]_2$, and $[Zn(tolf)_2(H_2O)]$ were prepared by the reaction of tolfenamic acid, a potent anti-inflammatory drug, with metal salts. The radical-scavenging activities of the complexes were evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assay. Their ability to inhibit soybean lipoxygenase, β-glucuronidase, and trypsin-induced proteolysis was studied. Their inhibitory effects on rat paw edema induced by carrageenin was studied and compared with those of tolfenamic acid. The complex $[Zn(tolf)_2(H_2O)]$ exhibited the strongest *in vivo* inhibitory effect at 0.1 mm/kg Body Weight (BW; $93.0\pm0.9\%$), superior than the inhibition induced by tolfenamic acid at the same molar dose ($76.0\pm0.9\%$). Tolfenamic acid and its metal complexes have been evaluated for antiproliferative activity *in vitro* against the cells of three human cancer cell lines, MCF-7 (breast cancer cell line), T24 (bladder cancer cell line), and A-549 (non-small cell lung carcinoma), and a mouse fibroblast L-929 cell line. The complexes $[Mn(tolf)_2(H_2O)_2]$ and $[Cu(tolf)_2(H_2O)]_2$ have shown selectivity against T24 cell line. The IC_{50} values of these two complexes against T24 cancer cell lines are in a micromolar range similar or better to that of the antitumor drug cisplatin.

Introduction. – Tolfenamic acid (=2-[(2-methyl-3-chlorophenyl)amino]benzoic acid; Htolf) is a potent, well-tolerated non-steroidal anti-inflammatory drug (NSAID) with a low gastroulcerogenicity [1], a low overall toxicity, and high therapeutic indices. It possesses analgesic and antipyretic properties as demonstrated in several animal models and has shown good results in long-term treatment of rheumatoid arthritis and osteoarthritis [2]. Chemically, it resembles mefenamic and flufenamic acids, other fenamates in clinical use. The anti-inflammatory activity of NSAIDs and most of its other pharmacological effects are related to the inhibition of the conversion of arachidonic acid (AA) to prostaglandins, which are mediators of the inflammatory process. The enzymes cyclooxygenase (COX) and lipoxygenase (LOX), which catalyze the oxidative metabolism of amino acids, are useful targets for the design and the development of new drugs that substantially inhibit the generation of the final inflammatory products and the propagation of inflammation. Recent studies revealed

that, in addition to arthritis and pain, cancer and neurodegenerative diseases like *Alzheimer*'s disease could potentially be treated with Cox-2 inhibitors [3][4].

Synthesis and study of metal complexes with active drugs as ligands, as an approach to new drug development [5][6], is a research area of increasing interest in inorganic, pharmaceutical, and medicinal chemistry. X-Ray crystal-structure investigations of Htolf revealed the existence of two polymorphic forms containing the molecule as different conformers [7] (*Fig. 1*). The crystal structure of dimeric tetraorganodistannoxane adducts of Htolf has been reported by our group [8]. We also recently reported synthesis, crystal structure, IR- and UV/VIS-spectroscopic data, and electrochemical studies of Cu^{II} complexes of Htolf (*Fig. 2*) [9]. The superoxide dismutase activity was measured, and IC_{50} value was determined by the *Fridovich* test. According to the usual criteria, the Cu complex of tolfenamic is considered as potent SOD mimic [9]. A review of the synthesis and crystal structures of a number of anti-inflammatory compounds as ligands in organotin complexes, and a review of Cu complexes with NSAIDs have recently been published [10][11].

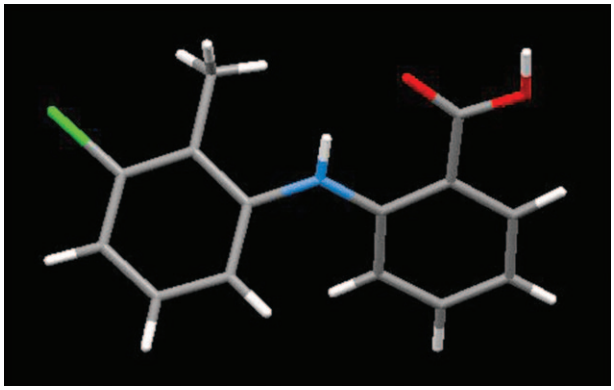
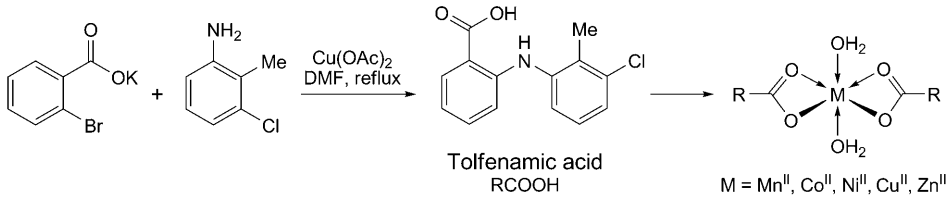


Fig. 1. *The structure of tolfenamic acid* [7]

We have synthesized and characterized novel complexes of Mn^{II}, Co^{II}, Ni^{II}, Cu^{II}, and Zn^{II} with Htolf, *i.e.*, $[Mn(tolf)_2(H_2O)_2]$, $[Co(tolf)_2(H_2O)_2]$, $[Ni(tolf)_2(H_2O)_2]$, $[Cu(tolf)_2(H_2O)]_2$, and $[Zn(tolf)_2(H_2O]$, and the complexes were tested for their antioxidant and anti-inflammatory activities in the carrageenin-induced rat paw edema assay. Also, their inhibition of LOX, of β-glucuronidase, and of trypsin-induced proteolysis was evaluated. The cytotoxic activity of Htolf and its metal complexes have been evaluated for antiproliferative activity *in vitro* against the cells of three human cancer cell lines, MCF-7 (human breast cancer cell line), T24 (bladder cancer cell line), and A-549 (non-small cell lung carcinoma), and a mouse cell line, L-929 (a fibroblast-like cell line cloned from strain L).

Results and Discussion. – 1. *Chemistry.* Tolfenamic acid (Htolf) was synthesized according to a published procedure, the *Ullmann–Goldberg* condensation [12] (*Scheme*). The complexes $[Mn(tolf)_2(H_2O)_2]$, $[Co(tolf)_2(H_2O)_2]$, $[Ni(tolf)_2(H_2O)_2]$, $[Cu(tolf)_2(H_2O)]_2$, and $[Zn(tolf)_2(H_2O)]$ were synthesized according to the *Reactions 1–5*, in MeOH and aqueous solutions.

Scheme. *Synthesis of Tolfenamic Acid and Its Metal Complexes*



$$MnCl_2 + 2\ Htolf \rightarrow [Mn(tolf)_2(H_2O)_2] + 2\ HCl \quad (1)$$

$$Co(AcO)_2 \cdot 4\ H_2O + 2\ Htolf \rightarrow [Co(tolf)_2(H_2O)_2] + 2\ AcOH + 2\ H_2O \quad (2)$$

$$NiCl_2 \cdot 6\ H_2O + 2\ Htolf \rightarrow [Ni(tolf)_2(H_2O)_2] + 2\ HCl + 4\ H_2O \quad (3)$$

$$Cu(AcO)_2 \cdot H_2O + 2\ Htolf \rightarrow [Cu(tolf)_2(H_2O)]_2 + 2\ AcOH \quad (4)$$

$$ZnCl_2 + 2\ Htolf \rightarrow [Zn(tolf)_2(H_2O)] + 2\ HCl \quad (5)$$

The complexes are microcrystalline or powder-like, and stable under atmospheric conditions. All the complexes are insoluble in H_2O and MeCN, and are soluble in non-polar solvents as benzene, toluene, $CHCl_3$, CH_2Cl_2, acetone, and THF, and polar solvents as DMF and DMSO. The elemental analyses confirm their stoichiometry. The μ_{eff} values of the complexes show that these are all high-spin; the large and small orbital contribution in the $[Co(tolf)_2(H_2O)_2]$ and $[Ni(tolf)_2(H_2O)_2]$, *i.e.*, $t_{2g}^5eg^2$ Co^{II} and $t_{2g}^6eg^2$ Ni^{II}, respectively, indicate six-coordinate structures.

2. *Spectroscopy.* 2.1. *IR Spectroscopy.* As the carboxy H-atom is more acidic than the amino H-atom the deprotonation occurs in the COOH group. This is confirmed by the IR spectra of the complexes, showing the characteristic bands for the secondary amino groups and for the coordinated carboxylato group. A broad absorption at 3400 cm^{-1} in the spectra of the complexes was attributed to the presence of coordinated H_2O. The absence of large systematic shifts of the $\tilde{\nu}(NH)$ and $\delta(NH)$ bands in the spectra of the complexes compared with those of the ligand indicates that there is no interaction between the NH group and the metal ions. The $\tilde{\nu}_{as}(COO)$ and $\tilde{\nu}_{sym}(COO)$ bands of the prepared complexes are at 1615–1580 and at 1500–1450 cm^{-1} respectively; The difference $\Delta(\tilde{\nu}_{as}(COO) - \tilde{\nu}_{sym}(COO))$ for the Mn, Co, Ni, and Zn complexes is less than the ionic value (for sodium tolfenamate, the Δ value is 160 cm^{-1}), as expected for the bidentate chelating mode of carboxylate ligation [8][9][13][14]. The difference, $\Delta(\tilde{\nu}_{as}(COO) - \tilde{\nu}_{sym}(COO))$ for the Cu complex is close and less (161 cm^{-1}) than for sodium tolfenamate, as expected for bridging bidentate carboxylato groups, supporting a structure analogous to $[Cu(tolf)_2(DMF)]_2$ (*Fig. 2*) [9] and $[Cu(diclof)_2(DMF)]_2$ [13]. The four carboxylate groups from four ligands are in a bidentate bridging mode. The square-pyramidal geometry with a DMF O-atom or H_2O occupying both apical positions as was established by single-crystal X-ray study [9][12]. The medium bands at *ca.* 400 cm^{-1} are attributed to the $\tilde{\nu}(M-O_{H_2O})$ stretching mode, while the bands at 250–210 cm^{-1} to the $\tilde{\nu}(M-O_{oco})$ stretching mode [8][9][12][13].

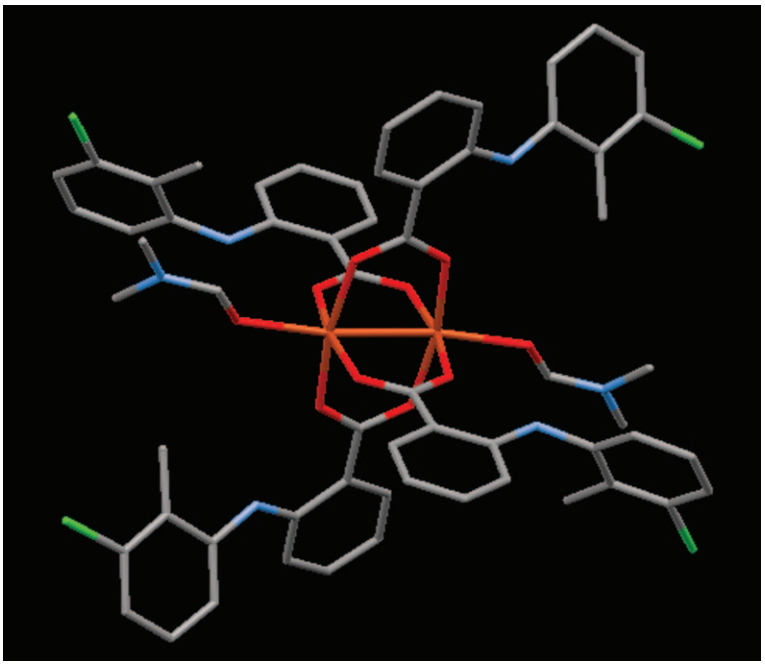


Fig. 2. *The structure of the complex [Cu(tolf)$_2$(DMF)]$_2$.* The H-atoms are omitted for clarity [9].

2.2. *Electronic Spectroscopy.* The electronic spectra were recorded in DMF and $CHCl_3$ solution, and absorption maxima in the UV/VIS region are listed in *Table 1* along with suggested assignments [9][13–15]. The electronic spectra of the complex $[Mn(tolf)_2(H_2O)_2]$ can be assigned to six-coordinate configuration [14][15]. The absorption of the organic ligand tailing into the visible region obscures the very weak d-d absorption bands of the Mn^{II} complexes. The d-d spectra of the solvated Mn, Co, and Ni complexes can be assigned to transitions in pseudo-octahedral structures or six-coordinated tetragonally distorted configurations. The band frequencies and the 10 Dq value in $[Ni(tolf)_2(H_2O)_2]$ are characteristic of a $Ni^{II}O_6$ chromophore, which do not cause a pronounced nephelauxetic effect [14][15]. The intense bands of complex of Co^{II} in the visible region, indicates a distorted octahedral configuration [14]. The solution spectral data indicate that the Mn, Co, and Ni complexes are all solvated with two solvent molecules coordinating in a pseudo-octahedral arrangement (*Reaction 6*).

$$[M(tolf)_2(H_2O)_2] + 2\ \text{solvate} \rightarrow [M(tolf)_2(\text{solvate})_2] + 2\ H_2O \qquad (6)$$

where M is Mn^{II}, Ni^{II}, or Co^{II}, and solvate is DMF or $CHCl_3$.

3. *Biological Studies.* The metal complexes and the parent drug were studied with regard to their radical-scavenging ability as well as to the inhibition of *a*) soybean lipoxygenase, *b*) β-glucuronidase, and *c*) trypsin-induced proteolysis.

The reducing abilities of the tolf complexes were evaluated using the 1,1-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging assay [15] in an iron-free system. This

Table 1. *Spectral Data* (UV/VIS) *and* μ_{eff} *Values of the Prepared Complexes and the Parent Drug*

	μ_{eff}	UV ($CHCl_3$)		UV (DMF)		Assignments
		λ [nm]	log ε	λ [nm]	log ε	
Tolfenamic acid		352 (sh)	3.73	347	3.82	$n \rightarrow \pi^*$
		288	3.81	295	4.16	$\pi \rightarrow \pi^*$
$[Mn(tolf)_2(H_2O)_2]$	5.96	547 (sh)		551 (sh)		$^6A_{1g} \rightarrow {}^4T_{1g}$ (G)
		351	4.30	337 (sh)	4.14	d-d , $n \rightarrow \pi^*$
		288	4.42	299	4.20	$\pi \rightarrow \pi^*$
$[Co(tolf)_2(H_2O)_2]$	4.88	580	1.97	560	1.69	$^4T_{1g} \rightarrow {}^4A_{2g}$
		531	1.91	538	1.70	$^4T_{1g} \rightarrow {}^4T_{1g}$ (P)
		351	3.94	331 (sh)	4.01	$n \rightarrow \pi^*$
		290	4.05	300	4.46	$\pi \rightarrow \pi^*$
$[Ni(tolf)_2(H_2O)_2]$	3.47	770 (sh)	0.91	750 (sh)	0.81	$^3A_{2g} \rightarrow {}^3T_{1g}$
		683	1.00	677	0.89	$^3A_{2g} \rightarrow {}^1E_g$
		343	4.33	338 (sh)	4.44	$n \rightarrow \pi^*$
		291	4.42	297	4.76	$\pi \rightarrow \pi^*$
$[Cu(tolf)_2(H_2O)]_2$	1.58	693	2.20	723	2.22	d-d
		351	3.98	330 (sh)	4.04	$n \rightarrow \pi^*$
		288	4.09	299	4.77	$\pi \rightarrow \pi^*$

interaction expresses the reducing ability of the compounds. The scavenging activities of the complexes were measured, and compared with those of the free drug and of vitamin C. The scavenging ability of the stable DPPH radical is extensively used as a model. DPPH is a stable free radical that can accept an electron or hydrogen radical, and thus be converted into a stable, diamagnetic molecule. DPPH has an odd electron and so has a strong absorption band at 517 nm. When this electron becomes paired off, the absorption decreases stoichiometrically with respect to the number of electrons taken up. Such a change in the absorbance produced in this reaction has been widely applied to test the capacity of numerous molecules to act as free-radical scavengers. Not all of the tested compounds were found to interact with the stable free radical DPPH (*Table 2*). Tolfenamic acid did not interact with the stable free radical. The Co^{II}, Cu^{II}, and Zn^{II} complexes present low interactions (3.9–27.2%). It seems that interaction increases with the time and the concentration. The Ni^{II} complex exhibits mild interaction (27.4–44.1%), and the complex $[Mn(tolf)_2(H_2O)_2]$ presents the highest interaction with DPPH, which is time- and concentration-dependent. Antioxidants that exhibit DPPH radical-scavenging activity is increasingly receiving attention. They have been reported to have interesting anticancer, anti-aging, and anti-inflammatory activities, and could offer protection in rheumatoid arthritis and inflammation, and lead to potentially effective drugs. In fact, many NSAIDs have been reported to act either as inhibitors of free-radical production or as radical scavengers.

During the inflammatory process, phagocytes generate the superoxide anion radical at the inflamed site, and this is connected to other oxidizing species such as HO•. Hydroxyl radicals are produced by reactions which are dependent on transition metals, particularly iron [16]. The competition of the compounds (0.1 mM) with DMSO for HO• generated by the Fe^{3+}/ascorbic acid system, expressed as the inhibition of formaldehyde production, was used for the evaluation of their HO• scavenging activity.

Table 2. *Interaction with DPPH* (scavenging activity (SA) [%])[a])

	0.1 mM		0.5 mM	
	20 min	60 min	20 min	60 min
$[Mn(tolf)_2(H_2O)_2]$	38.6	48.1	45.3	**56.8**
$[Co(tolf)_2(H_2O)_2]$	13.9	17.5	18.5	27.2
$[Ni(tolf)_2(H_2O)_2]$	27.4	36.9	27.4	44.1
$[Cu(tolf)_2(H_2O)]_2$	7.9	13.8	16.4	22.9
$[Zn(tolf)_2(H_2O)]$	3.9	11.5	3.4	20.4
$MnCl_2$	4.2	4.1	3.2	3.5
$CoCl_2$	3.1	2.7	2.7	2.9
$NiCl_2 \cdot 6\,H_2O$	3.1	6.1	5.1	4.9
$CuCl_2$	4.6	5.6	4.9	5.8
$ZnCl_2$	3.5	5.2	3.8	5.3
Ascorbic acid	**99.5**	**99.9**	**94.5**	**93.9**
Tolfenamic acid	2.6	2.8	4.4	8.9

[a]) Each experiment was performed at least in triplicate, and the standard deviation of absorbance was less than 10% of the mean.

In these experiments (*Table 3*), Htolf and its Mn^{II} and Co^{II} complexes show low inhibition, while complexes $[Ni(tolf)_2(H_2O)_2]$, $Cu(tolf)_2(H_2O)]_2$, and $[Zn(tolf)_2(H_2O)]$ exhibit very high competition (77–85%). Trolox was used as a reference compound.

Table 3. *Competition with DMSO* (0.1 mM) *for HO•, Inhibition of LOX, and Antiproteolytic Activity of Tolfenamic Acid and Its Metal Complexes*[a])

	Competition with DMSO (0.1 mM) [%]	Inhibition of		
		LOX at 5 min (0.033 mM) [%]	glucuronidase (0.1mM) [%]	trypsin (0.3 mM) [%]
Tolfenamic acid	16.9	no	**86.7**	25.1
$[Mn(tolf)_2(H_2O)_2]$	34.9	55.1	**88.8**	71.3
$[Co(tolf)_2(H_2O)_2]$	16.5	31.1	no	34.2
$[Ni(tolf)_2(H_2O)_2]$	77.8	58.7	**91.8**	0.2
$[Cu(tolf)_2(H_2O)]_2$	77	no	[b])	no
$[Zn(tolf)_2(H_2O)]$	**85.1**	**80.8**	**84**	48
$MnCl_2$	no	–	–	26
$CoCl_2$	no	–	–	
$NiCl_2 \cdot 6\,H_2O$	no	–	–	27
$CuCl_2$	no	–	–	75
$ZnCl_2$	no	–	–	24
Salicylic acid			56.4	[c])
Trolox	88.2			

[a]) Each experiment was performed at least in triplicate, and the standard deviation of absorbance was less than 10% of the mean. [b]) Not measured under the reported experimental conditions; no result. [c]) $IC_{50} = 0.1$ mM.

Most of the recognized LOX inhibitors are antioxidants or free-radical scavengers since lipoxygenation occurs *via* a C-centered radical. The activity of Htolf and its metal complexes to inhibit the enzyme soybean LOX has been studied. Htolf and its Cu^{II} complex did not exhibit inhibition of the enzyme LOX, while the Co^{II} complex shows low inhibition (31%). The Mn^{II}, Ni^{II}, and Zn^{II} complexes are potent LOX inhibitors (55–81% inhibition). The complex $[Zn(tolf)_2(H_2O)]$ presents the highest activity (*Table 3*).

The role played by proteases in the early stage of inflammatory process is well-documented. Some anti-inflammatory agents have been reported to exhibit antiproteolytic activity [16]. The antiproteolytic activity of the metal complexes and the parent drug was studied. Htolf and its Mn^{II}, Ni^{II}, and Zn^{II} complexes were found to inhibit significantly β-glucuronidase, while the Co^{II} complex did not show any effect against this enzyme under the reported experimental conditions. Complexes of Ni^{II} and Cu^{II} were found inactive against the enzyme trypsin. Htolf and its Co^{II} and Zn^{II} complexes present low effect (25–48% inhibition). The complex $[Mn(tolf)_2(H_2O)_2]$ was found to inhibit significantly the enzyme trypsin 71.3% (*Table 3*).

The *in vivo* anti-inflammatory effects of Htolf and its metal complexes were assessed by using the functional model of carrageenin-induced rat paw edema, and they are presented in *Table 4* as percentage of weight increase at the right hind paw in comparison to the un-injected left hind paw. Carrageenin-induced edema is a nonspecific inflammation resulting from a complex of diverse mediators [17]. Since edemas of this type are highly sensitive to NSAIDs, carrageenin has been accepted as a useful agent for studying new anti-inflammatory drugs [18]. This model reliably predicts the anti-inflammatory efficacy of the NSAIDs, and, during the second phase, it detects compounds that are anti-inflammatory agents, as a result of inhibition of prostaglandin amplification [19]. In *Table 4*, the protection, observed against carrageenin-induced paw edema, by the investigated complexes is shown. The reference drug, Htolf induced 76.0% protection at an equivalent concentration (tests were performed at 0.1 mmol/kg Body Weight (BW)). The anti-inflammatory activity of Htolf, and of the complexes $[Co(tolf)_2(H_2O)_2]$ and $[Zn(tolf)_2(H_2O)]$ on carrageenin-induced edema revealed statistically significant anti-inflammatory activity. The complex $[Zn(tolf)_2(H_2O)]$ exhibited a strong inhibitory effect at 0.1 mmol/kg BW (93.0 ± 0.9% inhibition), superior to the inhibition induced by Htolf at the same dose (76.0 ± 0.8% inhibition) on carrageenin-induced edemas. Zn has been reported to give

Table 4. *Inhibition of Carrageenin-Induced Rat Paw Edema* (CPE)[a])

	CPE ± SD [%][b])
Tolfenamic acid	76* ± 0.8
$[Co(tolf)_2(H_2O)_2]$	82.6** ± 0.8
$[Zn(tolf)_2(H_2O)]$	**93** ± 0.9**

[a]) The complexes have been tested at 0.1 mmol/kg. [b]) The effect on edema is expressed as percent of weight increase of hind paw (and as percent of inhibition of edema) in comparison to controls. Each value represents the mean obtained from six to eight animals in two independent experiments. In all cases, significant difference from control; *: $p < 0.1$; **: $p < 0.01$ (*Student*'s *t*-test).

potent anti-inflammatory complexes with other NSAIDs (*e.g.*, *Naproxen*). The observed differences in activity seem to depend on the nature of the metal.

The results of cytotoxic activity *in vitro* are expressed as IC_{50}, *i.e.*, the concentration of compound (in μM) that inhibits a proliferation rate of the tumor cells by 50% as compared to control untreated cells (*Table 5*). Htolf and its metal complexes were tested for their antiproliferative activity *in vitro* against the cells of three human cancer cell lines, MCF-7 (breast cancer cell line), T24 (bladder cancer cell line), and A-549 (non-small-cell lung carcinoma), and a mouse fibroblast L-929 cell line, and the results are compared with those of the known chemotherapeutic cisplatin.

Table 5. *The Antiproliferative Activity* in vitro *of Tolfenamic Acid and Its Metal Complexes* (expressed as IC_{50} [μM]) *against MCF-7, T-24, A-549, and L-929 Cancer Cell Lines*

	MCF-7	T-24	A-549	L-929
Tolfenamic acid	87.9 ± 6.3	62.4 ± 5.1	145 ± 12	214 ± 18
$[Mn(tolf)_2(H_2O)_2]$	41.6 ± 2.9	**3.9** ± 0.3	65.3 ± 6.1	149 ± 6
$[Co(tolf)_2(H_2O)_2]$	44.9 ± 3.0	38.3 ± 1.9	76.8 ± 7.1	33.4 ± 2.8
$[Ni(tolf)_2(H_2O)_2]$	54.5 ± 3.3	35.30 ± 2.1	54.1 ± 4.9	100.1 ± 9.1
$[Cu(tolf)_2(H_2O)]_2$	26.1 ± 2.1	**13.9** ± 1.1	31.4 ± 2.2	**5.3** ± 0.5
$[Zn(tolf)_2(H_2O)]$	41.3 ± 3.5	41.0 ± 2.5	57.9 ± 4.2	123 ± 6
$MnCl_2$	161 ± 11	93.5 ± 5.9	n.a.	n.a.
$CoCl_2$	n.a.[a])	91.4 ± 7.3	n.a.	n.a.
$NiCl_2$	n.a.	108 ± 9	n.a.	n.a.
$CuCl_2$	337 ± 22	96.7 ± 8.7	493 ± 3	n.a.
$ZnCl_2$	n.a.	n.a.	281 ± 20	35.0 ± 3.1
Cisplatin	8.0 ± 0.8	41.7 ± 4.5	1.5 ± 0.1	0.7 ± 0.1

[a]) n.a.: Not available.

The IC_{50} value for Htolf against the T24 cell line is 62.4 μM, and the IC_{50} values for the complexes $[Cu(tolf)_2(H_2O)]_2$ and $[Mn(tolf)_2(H_2O)_2]$ are 13.89 and 3.91 μM, respectively. In the case of T-24 cell line, $[Mn(tolf)_2(H_2O)_2]$ and $[Cu(tolf)_2(H_2O)]_2$ compared to cisplatin are 10.7 and 3.0 times, respectively, more cytotoxic, while Htolf is 1.5 time less cytotoxic than cisplatin. The complex $[Cu(tolf)_2(H_2O)]_2$, against L-929 cancer cell lines, is 7.5 times less cytotoxic than cisplatin. The complexes $[Mn(tolf)_2(H_2O)_2]$ and $[Cu(tolf)_2(H_2O)]_2$ have shown selectivities against T24 cell line, and the complex $[Cu(tolf)_2(H_2O)]_2$ against L-929 cell line. The *in vitro* results revealed that Htolf and the metal chlorides are not active or less active than the corresponding metal complexes of Htolf, indicating that the mechanism of action should be different.

The cytotoxic activities shown by $[Mn(tolf)_2(H_2O)_2]$ and $[Cu(tolf)_2(H_2O)]_2$ against T24 cancer cell line indicate that coupling of Htolf to Cu^{II} and Mn^{II} centers results in complexes with important biological properties, since they display IC_{50} values in a molar range similar or better to that of the antitumor drug cisplatin.

The reported therapeutic action of these metal complexes could be either due to a small proportion of metal complexes that can bind to biomolecules and can be transported to the site of action, or to a ternary metal–tolf–biomolecule complex which is also the transporting agent [20].

Conclusions. – The complexes of Htolf, *i.e.*, $[Mn(tolf)_2(H_2O)_2]$, $[Co(tolf)_2(H_2O)_2]$, $[Ni(tolf)_2(H_2O)_2]$, $[Cu(tolf)_2(H_2O)]_2$, and $[Zn(tolf)_2(H_2O)]$, were synthesized and characterized. Optical and IR-spectral data of these new complexes are reported. Monomeric six-coordinated species were isolated in the solid state for Mn^{II}, Ni^{II}, and Co^{II}, dimeric five-coordinated species for Cu^{II}, and monomeric five-coordinated species for Zn^{II}. In DMF or $CHCl_3$ solution, the coordination number is retained, and the coordinated molecules of H_2O are replaced by solvent molecules.

Htolf and its metal complexes have been studied with regard to their antioxidant ability, as well as their inhibition of LOX, of β-glucuronidase, and of trypsin-induced proteolysis. The complex $[Mn(tolf)_2(H_2O)_2]$ exhibits the highest antioxidant activity and a modest inhibitory effect against the soybean LOX, properties that are not demonstrated by Htolf. The same complex also exhibits a high inhibitory effect on β-glucuronidase and of trypsin-induced proteolysis. The anti-inflammatory effects of the complexes on rat paw edema induced by carrageenin were studied and compared with those of Htolf. The complex $[Zn(tolf)_2(H_2O)]$ did not interact with DPPH, whereas it exhibited a very high competition with DMSO for $HO^{\bullet}$ radical (85.1%), the highest inhibition against LOX enzyme (80.8%), and a high inhibition against the enzyme β-glucuronidase (84%). The complex $[Zn(tolf)_2(H_2O)]$ exhibited the strongest *in vivo* inhibitory effect at 0.1 mm/kg BW ($93.0 \pm 0.9\%$), superior to the inhibition induced by Htolf at the same molar dose ($76.0 \pm 0.9\%$). The IC_{50} value shown by $[Cu(tolf)_2(H_2O)]_2$ against the L-929 cancer cell lines are, in a molar range similar to cisplatin, 7.5 times less cytotoxic. The complexes $[Mn(tolf)_2(H_2O)_2]$ and $[Cu(tolf)_2(H_2O)]_2$ have shown selectivity against cell line T24. The IC_{50} values shown by these two complexes against cancer cell line T24 are, in a μM range, similar or better to that of cisplatin.

The aim of this research was to extend the pharmacological profile of Htolf, in order to search for new properties such as antioxidant and anticancer activity, to prepare new compounds, *i.e.*, complexes of Htolf with essential metal ions, which probably would exhibit improved or different biological properties compared to Htolf. The complex $[Zn(tolf)_2(H_2O)]$ may prove useful for treating inflammatory diseases, and the complexes $[Mn(tolf)_2(H_2O)_2]$ and $[Cu(tolf)_2(H_2O)]_2$ may lead to the development of an antitumor drug.

Experimental Part

1. *General.* The reagents (*Aldrich* and *Merck*) were used as supplied, while the solvents were purified according to standard procedures. Trypsin (pancreas protease) 200 Fip U/g, salicylic acid (SA), *N*-tosylarginine methyl ester (TAME), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and nordihydroguairetic acid (NDGA) were purchased from *Aldrich Chemical Co.* Milwaukee, WI, (USA). Soybean LOX and linoleic acid sodium salt were obtained from *Sigma Chemical, Co.* (St. Louis, MO, USA). Carrageenin, type K, is commercially available. M.p.: in open capillaries; uncorrected. UV Spectra: *Jasco V-570* spectrophotometer UV/VIS/NIR; λ in nm. IR and far-IR spectra: *Perkin-Elmer Spectrum JX Fourier* transform spectrophotometer; with KBr pellets ($4000-400\ cm^{-1}$) and nujol mulls dispersed between polyethylene disks ($400-40\ cm^{-1}$); in cm^{-1}. Magnetic moments: at r.t. by the *Evans* method with mercuric tetrathiocyanocobaltate(II) as the susceptibility standard with a *Sherwood Scientific Magnetic Susceptibility Balance*; the effective magnetic moments (μ_{eff}) values calculated by using the *Curie* equation: $\mu_{eff} = 2.83(\chi_M^{corr})^{1/2}T^{1/2}$. C, H, and N analyses were carried out by the microanalytical service of the University of Ioannina.

2. *Preparations. Synthesis of Tolfenamic Acid* (*Htolf*). 2-[(2-Methyl-3-chlorophenyl)amino]benzoic acid (Htolf) was synthesized according to a published procedure, *i.e.*, the *Ullmann–Goldberg* condensation [12]. 3-Chloro-2-methylbenzenamine (0.074 mol), potassium 2-bromobenzoate (0.076 mol), 4-ethylmorpholine (0.076 mol), and 0.8 g of anh. $Cu(OAc)_2$ in 30 ml of distilled DMF under N_2 were refluxed at 145° for 4 h. To the resulting soln., 20 ml of distilled DMF and 30 ml of 12% HCl were added. The aq. layer was decanted, and MeOH was added. The solid was collected and recrystallized three times from acetone. Yield: 40%. M.p. 202°. IR (KBr): 3341 (br.; $\tilde{\nu}$(NH)), 1552 ($\tilde{\nu}_{as}$(COO)), 1434 ($\tilde{\nu}_{sym}$(COO)). Anal. calc. for $C_{14}H_{12}ClNO_2$ (261.7): C 64.26, H 4.62, N 5.35; found: C 64.25, H 4.70, N 5.20.

Synthesis of [Mn(tolf)$_2$(H$_2$O)$_2$]. A soln. of $MnCl_2$ (0.0504 g, 0.4 mmol) in MeOH (5.0 ml) was added to a soln. of Htolf (0.1976 g, 0.8 mmol) in MeOH (2.0 ml). Drops of a methanolic soln. of 1N NaOH were added until the apparent pH value was *ca.* 7. The mixture was stirred at r.t. for 1 h and cooled to 5° in a refrigerator for 4 h. The brown precipitate was collected by filtration and recrystallized from hot MeOH. The solid was washed with cold MeOH/H_2O 5:1 and dried *in vacuo* to afford $[Mn(tolf)_2(H_2O)_2]$. Yield: 14%. M.p. 280° (dec.). IR: 3414*m* ($\tilde{\nu}$(NH)), 1614*s* ($\tilde{\nu}_{asym}$(COO)), 1503*s* ($\tilde{\nu}_{sym}$(COO)), 1582*s* ($\tilde{\nu}_{asym}$(COO)), 1459*s* ($\tilde{\nu}_{sym}$(COO)), 444*m–s*, 413*m–w* ($\tilde{\nu}$(M–O_{H_2O})), 382*ms*, 314*ms* ($\tilde{\nu}$(M–O_{oco})). μ_{eff} = 5.96 MB. Anal. calc. for $C_{28}H_{26}Cl_2MnN_2O_6$: C 54.92, H 4.28, N 4.58; found: C 54.45, H 4.19, N 4.62.

Synthesis of [Co(tolf)$_2$(H$_2$O)$_2$]. A soln. of $Co(OAc)_2 \cdot 4\,H_2O$ (0.0519 g, 0.4 mmol) in MeOH (5.0 ml) was added to a soln. of Htolf (0.1976 g, 0.8 mmol) in MeOH (5.0 ml). Drops of a methanolic soln. of 1N NaOH were added until the apparent pH value was *ca.* 7. The mixture was stirred at r.t. for 1 h and cooled to 5° in a refrigerator for 4 h. After the addition of a few drops of dist. H_2O, a pale pink powder precipitated. The pink precipitate was collected by filtration and recrystallized from hot MeOH. The powder was washed with cold MeOH/H_2O 5:1 and dried *in vacuo* to afford $[Co(tolf)_2(H_2O)_2]$. Yield: 34%. M.p. 145° (dec.). IR: 3335*s* ($\tilde{\nu}$(NH)), 1616*s* ($\tilde{\nu}_{asym}$(COO)), 1499*s* ($\tilde{\nu}_{sym}$(COO)), 1581*s* ($\tilde{\nu}_{asym}$(COO)), 1453*s* ($\tilde{\nu}_{sym}$(COO)), 420*m–w*, 397*m–w* ($\tilde{\nu}$(M–O_{H_2O})), 305*m*, 282*m* ($\tilde{\nu}$(M–O_{oco})). μ_{eff} = 4.88 MB. Anal. calc. for $C_{28}H_{26}Cl_2CoN_2O_6$: C 54.57, H 4.25, N 4.55; found: C 54.09, H 4.52, N 4.31.

Synthesis of [Ni(tolf)$_2$(H$_2$O)$_2$]. A soln. of $NiCl_2 \cdot 6\,H_2O$ (0.0951 g, 0.4 mmol) in MeOH (5.0 ml) was added to a soln. of Htolf (0.197 g, 0.26 mmol) in MeOH (5.0 ml). Drops of a methanolic soln. of 1N NaOH, were added until the apparent pH value was *ca.* 7. The mixture was stirred at r.t. for 1 h and cooled to 5° in a refrigerator for 4 h. After the addition of a few drops of dist. H_2O, a pale green powder precipitated. The precipitate was collected by filtration and recrystallized from hot MeOH. The powder was washed with cold MeOH/H_2O 5:1 and dried *in vacuo* to afford $[Ni(tolf)_2(H_2O)_2]$. Yield: 47%. M.p. 245° (dec.). IR: 3451 (br.; $\tilde{\nu}$(NH)), 1613*s* ($\tilde{\nu}_{asym}$(COO)), 1498 ($\tilde{\nu}_{sym}$(COO)), 1582*s* ($\tilde{\nu}_{asym}$(COO)), 1458 ($\tilde{\nu}_{sym}$(COO)), 467*m–w*, 444*m–w* (ν(M–O_{H_2O})), 324*m–w*, 280*m–w* ($\tilde{\nu}$(M–O_{oco})). μ_{eff} = 3.47 MB. Anal. calc. for $C_{26}H_{22}Cl_2N_2NiO_6$: C 52.81, H 4.26, N 4.19; found: C 53.07, H3.77, N 4.76. Anal. calc. for $C_{28}H_{26}Cl_2N_2NiO_6$: C 54.59, H 4.25, N 4.55; found: C 54.27, H 4.07, N 4.26.

Synthesis of [Cu(tolf)$_2$(H$_2$O)]$_2$·2 H$_2$O. The complex $[Cu(tolf)_2(H_2O)]_2$ was prepared according to the procedure reported in [9]. Yield: 50%. M.p. 145° (dec.). IR: 3480*s* ($\tilde{\nu}$(H_2O)), 3414 ($\tilde{\nu}$(NH)), 1614*s* ($\tilde{\nu}_{asym}$(COO)), 1459*m* ($\tilde{\nu}_{sym}$(COO)), 434*m*, 406*m* ($\tilde{\nu}$(M–O_{H_2O})), 307*m–w*, 288*m–w* ($\tilde{\nu}$(M–O_{oco})). μ_{eff} = 1.59 MB. Anal. calc. for $C_{56}H_{60}Cl_4Cu_2N_4O_{12}$; C 53.81, H 4.83; Cu, 10.17, N 4.48; found: C 53.90, H 4.70, N 4.75.

Synthesis of [Zn(tolf)$_2$(H$_2$O)]. A soln. of $ZnCl_2$ (0.0545 g, 0.4 mmol) in MeOH (5.0 ml) was added to a soln. of Htolf (0.1976 g, 0.8 mmol) in MeOH (5.0 ml). Drops of a methanolic soln. 1N NaOH were added until the apparent pH value was *ca.* 7. The mixture was stirred at r.t. for 1 h. After the addition of a few drops of dist. H_2O, a light yellow powder precipitated. The soln. was cooled to 5° in a refrigerator for 4 h. The precipitate was collected by filtration and recrystallized from hot MeOH. The powder was washed with cold MeOH/H_2O 5:1 and dried *in vacuo* to afford $[Zn(tolf)_2(H_2O)]$. Yield: 35%. M.p. 250° (dec.). IR: 3337*s* ($\tilde{\nu}$(NH)), 1664*s* ($\tilde{\nu}_{asym}$(COO)), 1523*s* ($\tilde{\nu}_{asym}$(COO)), 1583*s* ($\tilde{\nu}_{asym}$(COO)), 1452*s* ($\tilde{\nu}_{sym}$(COO)), 420*m*, 397*m–w* ($\tilde{\nu}$(M–O_{H_2O})), 280*m–s* ($\tilde{\nu}$(M–O_{oco})). Anal. calc. for $C_{28}H_{24}Cl_2N_2O_5Zn$: C 55.61, H 4.00, N 4.63; found: C 55.15, H 3.89, N 4.37.

3. *Experiments* in vitro *and* in vivo [21]. In the *in vitro* assays, each experiment was performed at least in triplicate, and the standard deviation of absorbance was less than 10% of the mean.

Determination of the Radical-Scavenging Activity of the Tested Complexes Using Their Interaction with the Stable Radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH). To a soln. of DPPH (0.1 and 0.5 mM) in abs. EtOH an equal volume of the compounds dissolved in EtOH was added. As control, EtOH soln. was used. The concentrations of the solns. of the compounds were 0.1 and 0.5 mM. After 20 and 60 min at r.t., the absorbance was recorded at 517 nm.

Competition of the Tested Compounds with DMSO for HO· Radicals. The HO· radicals, generated by the Fe^{3+}/ascorbic acid system, were detected, according to *Nash*, by the determination of HCHO produced from the oxidation of DMSO. The reaction mixture contained EDTA (0.1 mM), Fe^{3+} (167 μM), DMSO (0.1 and 6.6 mM) in phosphate buffer (50 mM, pH 7.4), the tested compounds (1 mM), and ascorbic acid (10 mM). After 30 min of incubation (37°), the reaction was stopped with CCl_3COOH (17% (*w*/*v*)).

Soybean LOX Inhibition Study in vitro. *In vitro* study was conducted as reported previously. The tested compounds (0.033 and 0.333 mM) dissolved in EtOH were incubated at r.t. with sodium linoleate (0.1 mM) and 0.2 ml of enzyme soln. ($1/3 \times 10^4$ (*w*/*v*) in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded during 5 min and compared with the appropriate standard inhibitor (NDGA 0.1 mM, 83.7% inhibition; 1 mM 94.7% inhibition).

Inhibition of β-Glucuronidase and of Proteolysis. Compounds in acetate buffer (0.1 mM, pH 7.4) were tested against *β*-glucuronidase (0.1 ml of 1 U/ml) with 2.5 mM *p*-nitrophenyl-*β*-D-glucopyranosiduronic acid. After incubation at 37° for 30 min, 2 ml of 0.5N NaOH soln. was added to the mixture, and the absorbance of the mixture was measured at 410 nm. TAME was used as substrate for trypsin. The reaction mixture consisted of 0.75 ml buffer (0.1M *Tris*·HCl, pH 7.8, in 50% MeOH (*v*/*v*)) and 0.14 ml TAME (0.01M in 50% (*v*/*v*) MeOH). Compounds dissolved in 50% MeOH were added (0.3 mM). The reaction was started by addition of 0.1 ml trypsin (1 mg/ml 0.001N HCl). The increase in the absorbance at 256 nm was determined in the next 4 min.

Inhibition of the Carrageenin-Induced Edema [21]. Edema was induced in the right hind paw of *Fisher 344* rats (150–200 g) by the intradermal injection of 0.1 ml of 2% carrageenin in H_2O. Both sexes were used. Females pregnant were excluded. Each group was composed of 6–15 animals. The animals, which have been bred in our laboratory, were housed under standard conditions, and received a diet of commercial food pellets and water *ad libitum* during the maintenance, but they were entirely fasted during the experiment period. Our studies were in accordance with recognized guidelines on animal experimentation (Guidelines for the care and use of laboratory animals published by the Greek Government 160/1991, based on EU regulations 86/609). The tested compounds, 0.01 or 0.1 mmol/kg BW, were suspended in H_2O with a few drops of *Tween 80* and ground in a mortar before use, or dissolved in H_2O and were given intraperitoneally simultaneously. The rats were euthanized 3.5 h after carrageenin injection. The difference between the weight of the injected and uninjected paws was determined for each animal. The change in paw weight was compared with that in control animals (treated with H_2O) and expressed as a percent inhibition of the edema CPE % values (*Table 4*). CPE % Values are the mean from two different experiments with a standard error of the mean less than 10%.

Antiproliferative Assay in vitro [22–24]. *Compounds.* Test solns. of the compounds tested (1 mg/ml) were prepared by dissolving the substance in 100 μl of DMSO completed with 900 μl of tissue culture medium. Then, the tested compounds were diluted in culture medium to reach the final concentrations of 100, 50, 10, 1, and 0.1 μg/ml. The solvent (DMSO) in the highest concentration used in the test did not reveal any cytotoxic activity.

Cells. The established *in vitro* human cancer cell lines MCF-7 (breast cancer cell line), T24 (bladder cancer cell line), and A-549 (non-small-cell-lung carcinoma), and a mouse L-929 (a fibroblast-like cell line cloned from strain L) were applied. The cell lines are maintained in the Cell Culture Collection of the University of Ioannina. Twenty-four h before addition of the tested agents, the cells were plated in 96-well plates at a density of 10^4 cells per well. The T-24 and MCF-7 cells were cultured in D-MEM (Modified Eagle's Medium) supplemented with 1% antibiotic and 10% fetal calf serum. L-929 Cells were grown in *Hepes*-buffered RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (50 U/ml), and streptomycin (50 mg/ml). A-549 Cells were grown in F-12K *Ham*'s medium supplemented with 1% glutamine, 1% antibiotic/antimycotic, 2% $NaHCO_3$, and 10% fetal calf serum. The cell cultures were maintained at 37° in a humid atmosphere saturated with 5% CO_2. Cell number was counted by the

Trypan Blue dye exclusion method. MCF-7, L-929, and A-549 cells were determined by the sulforhodamine B assay [22], and T24 cells by the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium hydrobromide) assay.

SRB (*Sulforhodamine B*) *Assay.* The details of this technique were described by *Skehan et al.* [23]. The cytotoxicity assay was performed after 72-h exposure of the cultured cells to varying concentrations (from 0.1 to 100 μg/ml) of the tested agents. The cells attached to the plastic were fixed by gently layering cold 50% Cl_3CCOOH (TCA) on the top of the culture medium in each well. The plates were incubated at 4° for 1 h and then washed five times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained for 30 min with 0.4% sulforhodamine B dissolved in 1% AcOH. Unbound dye was removed by rinsing with 1% AcOH. The protein-bound dye was extracted with 10 mM unbuffered *Tris* base for determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate.

MTT (*Sigma*). This technique was applied for cytotoxicity screening against T-24 cells growing in suspension culture. An assay was performed after 72-h exposure to varying concentrations (from 0.1 to 100 mg/ml) of the tested agents. For the last 3–4 h of incubation 20 μl of MTT soln. was added to each well (stock soln.: 5 mg/ml). The mitochondria of viable cells reduce a pale yellow MTT to a navy blue formazan, so, if more viable cells are present in the well, more MTT will be reduced to formazan. When the incubation time was completed, 80 μl of the lysing mixture was added to each well (lysing mixture: 225 ml of DMF, 67.5 g of sodium dodecylsulfate (both from *Sigma*), and 275 ml of dist. H_2O). After 24 h, when formazan crystals had dissolved, the optical densities of the samples were read on an *Eliza SpectraMax 190* photometer at 570-nm wavelength. Each compound at a given concentration was tested in triplicate in each experiment, which was repeated three times [24].

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Received March 24, 2008