6-Alkoxymethyl-3-hydroxy-4H-pyranones: potential ligands for cell-labelling with indium

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Abstract. We have identified ligands for cell labelling with indium-111: 3-hydroxy-6-propoxymethyl-4H-pyran-4-one and 6-butoxymethyl-3-hydroxy-4H-pyran-4-one. The leucocyte labelling efficiencies of ^{111}In complexes of these ligands were higher and label stabilities were found to be similar compared with those obtained using ^{111}In-tropolonate. High labelling efficiencies of neutrophils and lymphocytes were achieved with ^{111}In complexes of pyranones. Tropolone was found to have a greater inhibitory effect on metalloenzymes and to cause greater impairment of platelet function than 3-hydroxy-6-propoxymethyl-4H-pyran-4-one. Thus 6-alkoxymethyl-3-hydroxy-4H-pyran-4-ones may have advantages over current ligands used in cell labelling with ^{111}In.

Key words: Hydroxypyranones – Indium – Radiolabelling – Leucocytes – Platelets

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Introduction

The radiolabelling of blood cellular elements with indium-111 is a well-established procedure in nuclear medicine. Radiolabelled "mixed" leucocytes are used for diagnostic clinical investigations of non-quantitative imaging of inflammation and infection [1–4]. Cell kinetic and quantitative imaging studies require the use of neutrophils or granulocytes [5, 6]. Lymphocytes have been radiolabelled for lymphocyte kinetic and migration studies [7, 8]. ^{111}In-labelled platelets have been used for the imaging of thrombotic lesions and for platelet survival studies [9–12].

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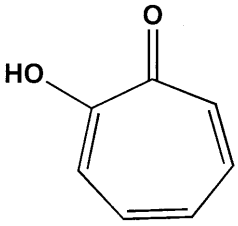
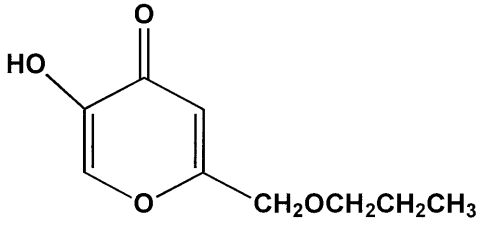
It is essential that labelled blood elements are not damaged by the cell-labelling agent, as this could affect the migration of leucocytes to sites of infection or inflammation as a result of an altered chemotactic response or cause an adverse effect on platelet function [13, 14]. The bidentate ligands oxine and tropolone have been widely used as cell-labelling agents with indium [15–18]. Both these agents form neutral 3:1 complexes with indium. This enables permeation of the cell membrane, followed by retention of the radionuclide in the cell, possibly by binding to cytoplasmic components [19]. Both oxine and tropolone have been reported to be toxic to cells, particularly lymphocytes [20–24].

As with oxine and tropolone, 3-hydroxypyranones and 3-hydroxypyridinones form 3:1 neutral complexes with trivalent cations at pH 7.4 [25]. 2-Ethyl- and 2-methyl-3-hydroxy-4H-pyran-4-ones (ethylmaltol and maltol respectively) have been used as flavouring and aroma agents in foodstuffs and possess a relatively low degree of toxicity [26, 27]. We have synthesized a range of 3-hydroxypyranones and 3-hydroxypyridinones with varying alkyl substitution, thereby influencing their hydrophobicity. The distribution coefficients, *D*, of these agents and their corresponding indium complexes have been determined (the ratio of distribution of compound or complex between *n*-octanol and aqueous buffer pH 7.4 is used as a measurement of lipophilicity) [28]. Clear structure-activity relationships were observed from which compounds with optimal cell labelling properties were identified. An ideal cell labelling agent should have a log *D* value >0 for the indium complex, thereby allowing permeation of the cell membrane, and a relatively low log *D* value for the free ligand, thereby minimizing partitioning in the membrane which may lead to toxic effects. Compounds possessing such properties were selected from the previous structure-activity study, namely 3-hydroxy-6-propoxymethyl-4H-pyran-4-one (**2**) and 6-butoxymethyl-3-hydroxy-4H-pyran-4-one (**3**) for cell labelling studies (Fig. 1).

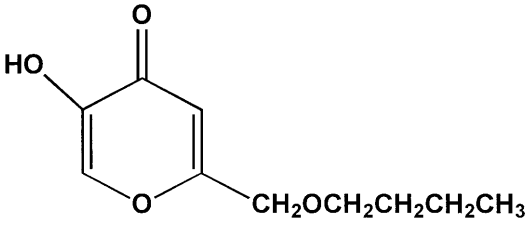
We report the labelling of "mixed" leucocytes, neutrophils and lymphocytes with the ^{111}In complexes of



1 (2-hydroxy-2,4,6-cycloheptatrienone or tropolone)



2 (3-hydroxy-6-propoxymethyl-4H-pyran-4-one)



3 (6-butoxymethyl-3-hydroxy-4H-pyran-4-one)

Fig. 1. Structures of 2-hydroxy-2,4,6-cycloheptatrienone (tropolone) (**1**), 3-hydroxy-6-propoxymethyl-4H-pyran-4-one (**2**) and 6-butoxymethyl-3-hydroxy-4H-pyran-4-one (**3**)

these heterocyclic bidentate chelators (**2** and **3**) and the evaluation of label stabilities. The effect of plasma on the uptake of ^{111}In complexes into erythrocytes and the influence of ligands on metalloenzymes and platelet function are reported.

Materials and methods

Ligands

3-Hydroxy-6-propoxymethyl-4H-pyran-4-one (**2**) and 6-butoxymethyl-3-hydroxy-4H-pyran-4-one (**3**) were synthesized by the method previously described by Ellis et al. [28].

Tropolone (**1**) (Fig. 1) was obtained from Aldrich Chemical Company (Dorset, UK) and as a 0.054% w/v solution in HEPES-saline buffer pH 7.6 from Pharmacy Manufacturing Department, Norfolk and Norwich Hospital, Norfolk, UK. $^{111}InCl_3$ in 0.04 *M* HCl (no carrier added) was purchased from Mallinckrodt Medical (Northampton, UK).

Separation of "mixed" leucocytes

Whole blood (60 ml) was collected from human volunteers into a 50-ml syringe containing 6 ml ACD (NIH, Formula A, Pharmacy Manufacturing Department, Norfolk and Norwich Hospital, Norfolk, UK). A 30 ml volume was transferred to two 50-ml Falcon tubes each containing 5 ml of Hespan (DuPont Pharma, Hertfordshire, UK). After sedimentation of erythrocytes and centrifugation (80 *g* for 10 min) a pellet of mixed leucocytes was obtained. Centrifugation of the supernatant (1500 *g* for 10 min) resulted in platelet-poor plasma. The mixed leucocyte pellet was resuspended in platelet-poor plasma to a total volume of 3.3 ml. 800 µl of the cell suspension (3.3×10^7 cells) was transferred to each of three 10-ml Falcon tubes and the mixed leucocyte pellet was obtained by centrifugation.

Separation of neutrophils

Separation was achieved utilizing a method similar to that used by Sampson and Solanki, who have reported the purity of the cells to be 96% [29]. A 7.5 ml volume of a density gradient medium 1.119 g/ml (Histopaque 1119, Sigma Chemical Company, Dorset, UK) was dispensed into a 50-ml Falcon tube. 8.5 ml of a density gradient medium 1.077 g/ml (Histopaque 1077, Sigma Chemical Company, Dorset, UK) was carefully layered over the first density gradient medium. 15 ml of human blood was collected into a 30-ml syringe containing 1.5 ml ACD and poured onto the surface of the density gradient media, ensuring that the interface was not disrupted. The tube was centrifuged at 750 *g* for 15 min, resulting in the formation of two layers. The top layer was removed and aliquots dispensed into two 10-ml Falcon tubes and centrifuged to obtain pellets of neutrophils.

Separation of lymphocytes

Lymphocytes were isolated by a technique which has been reported to yield 81% purity [30]. 60 ml of human blood was collected by venipuncture into a syringe containing 6 ml of ACD. A 30 ml volume was transferred to two 50-ml Falcon tubes which each contained 5 ml of Hespan. After sedimentation and centrifugation the mixed leucocyte pellet was resuspended in 6 ml of platelet-poor plasma. The cell suspension was layered onto 3 ml of Histopaque 1077 (Sigma Chemical Company, Dorset, UK) and centrifuged at 400 *g* for 30 min. The lymphocyte layer was transferred to a 10-ml tube and aliquots were centrifuged to obtain lymphocyte pellets.

Cell-labelling

Ligand solutions (1×10^{-3} *M*) of **2** and **3** were prepared in phosphate-buffered saline pH 7.4 (Pharmacy Manufacturing Department, Norfolk and Norwich Hospital, Norwich, UK). The ^{111}In complexes of **1**, **2** and **3** were prepared by the addition of 1 ml of each ligand solution to 2 MBq of $^{111}InCl_3$. A 1 ml volume of the ^{111}In complexes was added to each cell pellet and incubated at room temperature for 15 min. After incubation the cells were washed with platelet-poor plasma and the labelling efficiencies were calculated.

Evaluation of label stability

A 3 ml volume of platelet-poor plasma was added to each of the radiolabelled cell pellets, which were incubated at room temperature for 1 h. After centrifugation and separation of the supernatants the percentage activity released from the cells was calculated. This process was repeated after 2, 3 and 4 h.

Cell viability studies

Mixed leucocytes were radiolabelled with the ^{111}In complexes of **1**, **2** and **3** as described above. Each radiolabelled cell aliquot was resuspended in 1 ml of platelet-poor plasma. In each case, cell viability was assessed by the addition of 80 µl of cell suspension to 20 µl of trypan blue solution. Non-viable cells will incorporate the trypan blue dye. Mixed leucocytes were labelled with the indium complexes of **1**, **2** and **3** as described above; the morphology of the cells was examined by microscopy and compared with leucocytes exposed to saline as a control.

Investigation of the percentage plasma concentration on uptake of ^{111}In into erythrocytes

30 ml of human blood was collected into each of two 50-ml syringes, each containing 3 ml ACD, with a 19-G needle. The anticoagulated blood was transferred to two 50-ml Falcon tubes each containing 5 ml of Hespan. After sedimentation, the red cells were washed and diluted with phosphate-buffered saline (PBS). 300-µl aliquots (1.9×10^9 cells) of cell suspension were added to Sterilin tubes and centrifuged at 1000 *g* for 10 min to obtain pellets of red cells. 0.2 ml, 0.5 ml, 0.75 ml and 1 ml of platelet-poor plasma were added to tubes containing the cell pellets. Tubes containing only the cell aliquots were used as controls. The radiolabelling of each cell aliquot with the ^{111}In complexes of **1**, **2** and **3** was performed as described above.

In vitro platelet aggregation studies

Whole blood was taken into citrate phosphate dextrose adenine 1 (CPDA-1) (7 parts blood to 1 part anticoagulant) [31]. The blood was centrifuged at 170 *g* for 20 min and the platelet-rich plasma (PRP) was obtained [32]. The hydroxypyranones (**2** and **3**) were prepared as 2 m*M* stock solutions in PBS pH 8. Tropolone (**1**) was prepared as a stock solution at a concentration of 30 m*M*. A volume of 450 µl of citrated PRP (250×10^9 l^{-1} platelets) was incubated with each chelator (300 µM) for 10 min at 37°C. Aggregation was induced using collagen (1 µg ml^{-1}). 270 µl of platelet suspension was prewarmed to 37°C under constant stirring (990 rpm) in an aggregometer. A volume of 30 µl of collagen was added and the maximal aggregatory response at 4 min was recorded.

Inhibition of soybean lipoxygenase studies

The effect of the chelators on the inhibition of soybean lipoxygenase was investigated using the method of Abeysinghe et al. [33]. Soybean lipoxygenase (sbLPO) (Sigma Chemical Company, Dorset, UK) was prepared as a 10,000 units ml^{-1} stock in 0.2 *M* borate buffer, pH 9.0 and linoleic acid (Sigma Chemical Company, Dorset, UK) as a 3.21 *M* stock solution in ethanol. Each chelator (300 µM) was pre-incubated with the enzyme (concentration 500 units/3.0 ml reaction volume) for 30 min. The reaction was initiated by the addition of linoleic acid (667 µ*M* in borate buffer). The inhibition of the enzyme was monitored by a change in the absorption of the product linoleic hydroperoxide at 234 nm in a 3.0-ml quartz cuvette. The rates of reactions in the absence or presence of chelators were determined using the molar absorption coefficient of the hydroperoxide (ε=23,600 mol^{-1} cm^{-1}).

Inhibition of DNA synthesis studies

The assessment of cellular DNA synthesis in the presence of the chelators was carried out using the method of Hoyes et al. [34]. Human K562 erythroleukaemia cells were cultured at 37 °C in RPMI 1640 medium with 5% (v/v) heat-inactivated fetal calf serum under CO_2:air (1:19). Viability was assessed by fluoresence microscopy using fluorescin di-acetate and ethidium bromide stains. Ethidium bromide is incorporated into dead cells which fluoresce with a red colour and fluorescin di-acetate is incorporated into viable cells which are observed by a green colour under UV light. The cells were incubated with each chelator (330 µ*M*) for 2 h, after which 200 µl of the cell suspensions were plated onto a microtitre plate and with 37 kBq [^{3}H]thymidine for 1 h. Cells were harvested onto glass fibre filters with an Automash 2000 (Dynex Technologies, Billingshurst, UK). Incorporation of the [^{3}H] label was measured by liquid scintillation counting.

Results

*Labelling efficencies of ^{111}In-complexes of **1**, **2** and **3** with mixed leucocytes and evaluation of label stability*

It was observed that there was a high uptake of ^{111}In into mixed leucocytes for all three ^{111}In complexes (Table 1).

Table 1. The percentage labelling efficiencies of ^{111}In complexes with leucocytes. The results are the mean of three observations ±SD

^{111}In complex	% Labelling efficiency		
	Mixed leucocytes	Neutrophils	Lymphocytes
1	76±6		
2	89±4	92±3	79±2
3	87±5	91±3	83±2

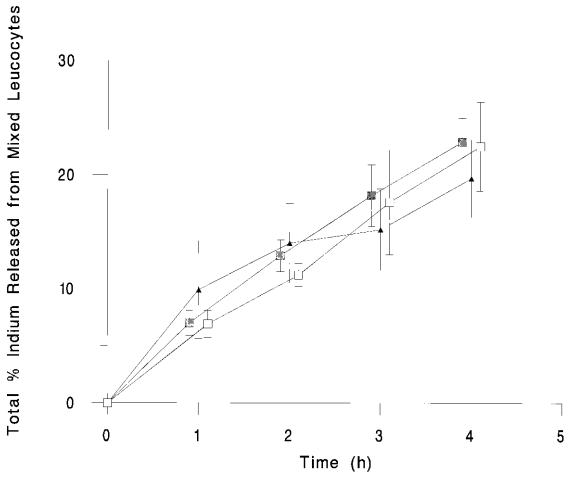


Fig. 2. The influence of time on the release of ^{111}In from mixed leucocytes labelled with the ^{111}In complexes of **1** (▲), **2** (■) and **3** (□). Mixed leucocytes labelled with the ^{111}In complexes were incubated with 3 ml of cell-free plasma for 1 h and the percentage ^{111}In released determined. The process was repeated for 2, 3 and 4 h. The mean of three independent readings is shown with the standard deviation

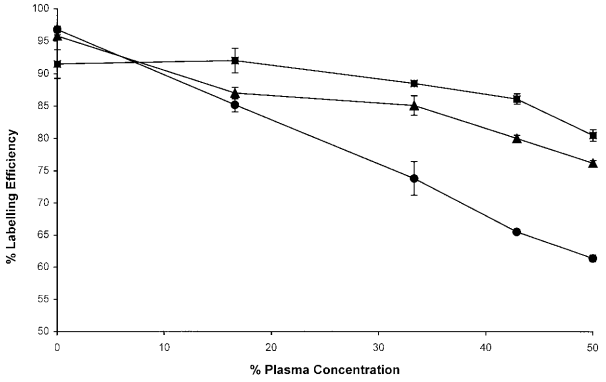


Fig. 3. The effect of plasma concentration on the uptake of the ^{111}In complexes of **1** (▲), **2** (■) and **3** (●) by erythrocytes. The mean of three observations is shown with standard error

The labelling efficiencies for the ^{111}In complexes of **2** and **3** are greater than that of ^{111}In-tropolonate (Student's *t* test $P<0.05$). As regards the label stability for the three ^{111}In complexes, the % ^{111}In released was not significantly different at any time point ($P>0.2$) (Fig. 2). Generally the amount of ^{111}In released in the first hour was greater than that released in subsequent hours.

Neutrophil and lymphocyte labelling and label stability

The % labelling efficiencies for the ^{111}In complexes of **2** and **3** with neutrophils and lymphocytes is presented in Table 1; as with mixed leucocytes, a high uptake of ^{111}In was observed. There was no significant difference between the labelling of neutrophils by ligand **2** or **3** ($P>0.4$). The label stabilities of ^{111}In-labelled neutrophils and lymphocytes, assessed by ^{111}In efflux, are presented in Table 2. For neutrophils, after an initial release of approximately 10%–13% of the label, only 6%–8% of the label was effluxed per hour. The ^{111}In complex of **3** showed slightly less efflux of ^{111}In than complex **2** over the time period, but this did not achieve significance in the accumulated efflux of ^{111}In until 4 h ($P<0.05$). For lymphocytes, more ^{111}In was effluxed from the lymphocytes during the first hour of incubation, and a similar amount released during the second hour. The accumulated ^{111}In efflux from lymphocytes was always greater with ligand **3** than ligand **2** but this was only significant at 1 h ($P<0.001$). With both cell types, approximately 30%–35% of the ^{111}In was released during the 4-h incubation period.

The effect of plasma concentration on the uptake of ^{111}In into erythrocytes

The effect of the plasma concentration on the uptake of the ^{111}In complexes of **1**, **2** and **3** into erythrocytes is presented in Fig. 3. For the three ^{111}In complexes a decrease in labelling efficiency is observed with an increase in plasma concentration. The decrease in the labelling efficiency of the ^{111}In complex of **3** is more pronounced than the decrease in that of ^{111}In complexes of **1** and **2**. At plasma concentrations of 50% there is a highly significant difference between the labelling efficiency of ^{111}In complex of **3** and that of ^{111}In complexes of **1** and **2** ($P<0.001$), and also a significant difference between the labelling efficiencies of the ^{111}In complexes of **1** and **2** ($P<0.01$) . Over the range of plasma concentration (0%–50%), the uptake of the ^{111}In complexes of **1** and **2** fall by approximately 20% and 10% respectively, as

Table 2. The total percentage ^{111}In released from neutrophils and lymphocytes radiolabelled with the ^{111}In complexes of **2** and **3**. The results are the mean of three observations ±SD

Cell type	^{111}In complex	Time (h)			
		1	2	3	4
Neutrophils	**2**	13.1±8.5	20.2±4.6	28.1±3.4	35.5±3.5
	3	10.7±3.1	17.0±0.9	22.9±3.4	29.0±2.8
Lymphocytes	**2**	9.0±0.3	17.8±2.9	24.9±3.2	30.7±7.5
	3	12.3±0.4	21.2±2.0	28.5±1.7	35.6±1.6

Table 3. The effect of tropolone (**1**) and pyranone (**2**) on the activity of Fe(III) metalloenzymes. The concentration of chelators were 300 μ*M* and 330 μ*M* for inhibition of soybean lipoxygenase and DNA synthesis studies respectively

Compound	% Inhibition of sbLPO (30 min)[a]	% Inhibition of DNA synthesis (2 h)[b]
1	63.6±4.6	16.0±1.7
2	41.2±5.0	5.6±1.5

[a] Mean±SE (n=4)
[b] Mean±SE (n=3)

compared to approximately 35% for the ^{111}In complex of **3**.

Cell viability studies

Cell viability studies using the trypan blue exclusion test with a mixed leucocyte population were undertaken for the ^{111}In complexes of **1**, **2** and **3**. The viabilities for **2** and **3** were assessed as 99% whereas for tropolone (**1**) the viability was assessed as 95%.

The morphology of the leucocytes after labelling with the indium complexes of **1**, **2** and **3** was examined by microscopy and compared with that of leucocytes in a control solution (saline). The leucocytes labelled with the indium complex of **1** showed differences in cell morphology compared with those exposed to the indium complexes of **2** and **3** and control solution (saline). Leucocytes labelled with the indium complex of **1** were asymmetric and ruffled, whereas leucocytes exposed to the indium complexes of **2** and **3** and the control were normal and spherical.

The chelating agents **1, 2** and **3** (300 μ*M*) produced aggregation responses for platelets which were 80± 15 SD, 121±8 SD and 117±13 SD percent of the control respectively. Thus, an impairment of aggregatory response was observed with tropolone (**1**) (P<0.001), in contrast to the pyranones **2** and **3** (P>0.2).

Metalloenzyme inhibition

The pyranones (**2** and **3**), like tropolone (**1**), bind iron(III) tightly [25]; consequently these ligands have the potential to inhibit iron-containing metalloenzymes. The inhibitory response of sbLPO after 30 min incubation with **1** and **2** (300 μ*M*) is shown in Table 3. The inhibition is greater with tropolone than with pyranone **2** (P<0.01).

Inhibition of DNA synthesis, which was used as an estimate of ribonuclease reductase inhibition, was determined by measuring the incorporation of [^{3}H]thymidine into K562 cells. The cell viability was found to be >85%. The percentage DNA inhibition after incubating the cells with each chelator for 2 h is shown in Table 3. A desferrioxamine control was used to provide an estimate of inhibition of de novo synthesis of ribonucleotide reductase [35]. Tropolone (**1**) was found to have a greater effect on DNA synthesis than pyranone (**2**). The percentage inhibition of DNA synthesis owing to **2** was less than that induced by **1** (P<0.01).

Discussion

An important requirement in the radiolabelling of leucocytes and platelets for the investigation of normal and pathological conditions is that the cells follow their natural behaviour when returned to the patient. If the leucocytes are damaged by the cell labelling agent, the chemotactic response may be altered, and this could affect the migration to sites of infection and inflammation. The bidentate ligands oxine and tropolone have been widely used for labelling cells with ^{111}In for over 15 years. However, the toxic effects of these compounds have been reported [20–24]. Bidentate ligands forming 3:1 neutral complexes with metal ions form part of a large family including the hydroxypyridinones and hydroxypyranones. In principle the properties of the hydroxypyranones could be optimized to generate a cell labelling agent with reduced toxicity while retaining the efficacy demonstrated by the currently used ligands oxine and tropolone.

In a systematic study of 22 bidentate ligands, three compounds with predicted optimal partition properties were identified [28]. These compounds were 3-hydroxy-6-propoxymethyl-4H-pyran-4-one (**2**), 6-butoxymethyl-3-hydroxy-4H-pyran-4-one (**3**) and tropolone (**1**), the free ligands of which possessed low log D values. The two pyranones (**2** and **3**) were found to be equi-effective with tropolone (**1**) at labelling leucocytes with ^{111}In as demonstrated by the uptake experiments with mixed leucocytes, neutrophils and lymphocytes and efflux experiments with the same cell types.

The bidentate ligands which have been used in clinical practice for radiolabelling leucocytes are non-selective for the cell types and will also bind to transferrin. In the case of ^{111}In-oxine if cells are labelled in a medium containing 90% plasma, the indium preferentially binds to plasma proteins, resulting in a labelling efficiency of only 5%. On the other hand if the cells are washed free of plasma and labelled in buffered saline, the labelling efficiency may be as high as 95% [1]. Even though this agent has been used in clinical practice for over 20 years, there has been a debate on whether depriving the cells of plasma may result in the cells becoming metabolically activated and the possibility that labelling cells in small amounts of plasma (10%) may have a protective effect on the cells [36]. The radiolabelling of cells in plasma is less affected by ^{111}In-tropolonate than by ^{111}In-oxine,

and cells may be labelled with the former in the presence of plasma. However, the labelling efficiency is lower than that achieved in buffered saline, presumably because of the competition between the plasma proteins and the cells for the ^{111}In. Thus the amount of ^{111}In incorporated into a fixed number of cells will decrease with an increase in plasma concentration [13]. A similar trend is observed with the uptake of the ^{111}In complexes of **1**, **2** and **3** into erythrocytes in the presence of varying amounts of plasma. The decrease in the labelling efficiency of the ^{111}In complex of **3** is more pronounced than the decrease in that of ^{111}In complexes of **1** and **2**. This study indicates that high labelling efficiencies may be achieved in the presence of small amounts of plasma (10%–20%) with the ^{111}In complex of **2**, which is comparable to that of ^{111}In-tropolonate. Thus the ligand **2** compares well in cell labelling properties with that of tropolone (**1**) and consequently was chosen for toxicity studies. Erythrocytes were chosen as the cell type to investigate the effect of plasma on the uptake of the ^{111}In complexes as they were easier to prepare and more readily obtainable for such a study.

We investigated the comparative toxicities of the pyranone **2** and tropolone (**1**) on blood cells. The cell viabilities of mixed leucocytes using the trypan blue exclusion test showed no appreciable difference between the two ligands. However, on microscopic examination of leucocytes labelled with the indium complexes of **2** and **1** appreciable differences in morphology were observed; the cells exposed to the former appeared to be normal and spherical whereas the appearance was asymmetrical and ruffled with the latter. Leucocytes may undergo some activation as a result of separation and labelling, however, this activation may be further increased by the presence of indium tropolonate whereas no such increase is observed with the indium complex of **2**.

Platelet aggregation studies indicate that short-term incubation with oxine has an adverse effect on platelet function [32]. The collagen-induced aggregation response of platelets was also observed with tropolone (**1**). In contrast the ligands **2** and **3** did not appear to impair platelet function. The inhibition of platelet aggregation by the bidentate chelating agents oxine and tropolone may be due to interaction with metal-containing enzymes, and in platelets cyclo-oxygenase is the main enzyme involved in the biosynthesis of thromboxane A_2. Thus agents which inhibit cyclo-oxygenase would be expected to have an anti-aggregatory effect. As metalloenzymes play a key role in cell function our work involved investigating the effects of the chelating agents **1** and **2** on the inhibition of soybean lipoxygenase and DNA synthesis. In both cases tropolone (**1**) was found to have a greater inhibitory effect on these metalloenzymes than ligand **2**.

Thus we conclude that 3-hydroxy-6-propoxymethyl-4H-pyran-4-one (**2**) offers some potential advantages over tropolone (**1**).

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