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A newly synthesized molecule derived from ruthenium cation, with antitumour activity, activates NADPH oxidase in human neutrophils

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To determine the nature of the mechanism by which certain derived ruthenium (Ru) complexes induce regression in tumour growth, we have investigated the possibility that this mechanism was associated with an increase of superoxide anion ($O_2^{-\bullet}$) production by phagocytic cells, which are usually found in tumour nodes. Here we present evidence that a newly synthesized complex, Ru^{3+}–propylene-1,2-diaminotetra-acetic acid (Ru–PDTA), derived from Ru and the sequestering ligand (PDTA), specifically stimulates $O_2^{-\bullet}$ production. This increase was associated with the translocation of cytosolic factors $p47^{phox}$ and $p67^{phox}$ of NADPH oxidase to the plasma membrane. The Ru–PDTA-complex-dependent $O_2^{-\bullet}$ production was abrogated by staurosporine, partially inhibited by diphenylene iodonium, and it was insensitive to pertussis toxin or dibutyryl cyclic AMP pretreatment. An increase of cytosolic Ca^{2+} levels were also detected in neutrophils treated with the Ru–PDTA complex. Also, Ru–PDTA complex induced the phosphorylation of tyrosine residues of several proteins as assessed by Western blotting. Present data are consistent with the possibility that Ru–PDTA-dependent antitumour effects are due in part to the complex's ability to stimulate the release of toxic oxygen metabolites from phagocytic cells infiltrating tumour masses.

INTRODUCTION

The potential use of ruthenium (Ru^{3+}) complexes in cancer chemotherapy has been recognized by researchers in different areas [1–4]. These substances have not only shown good anti tumour activity in screening studies [5,6], but many also localize in tumour tissues [7]. Ru^{3+} compounds, as prodrugs, can be reduced in the hypoxic tumoural areas into more active species which bind rapidly to cellular DNA [8,9]. In this way, amine–Ru complexes may be activated *in vivo* to co-ordinate the appropriate atoms of the nucleobase in a similar fashion to that of platinum anticancer drugs [10–12]. A recently investigated new group of promising anticancer compounds, namely Ru complexes with polyaminopolycarboxylic chelating ligands, are highly soluble in water and their active forms are six-co-ordinate with octahedral structures [13]. Recently we synthesized and characterized a new molecule formed by Ru^{3+} and the chelating ligand propylene-1,2-diaminetetra-acetic acid (PDTA) [14]. Compounds formed by this chelating agent, a methyl derivative of EDTA, are about two orders of magnitude more stable than the analogues formed with the parent ligand. It is likely that these complexes are transported to the tumour site by transferrin [15,16]. Indeed, the *in vivo* distribution of Ru might be determined by its interaction with this iron-transport protein because of similarities between the two metal ions. Since malignant cells have a high requirement for iron, it has been suggested that the accumulation of Ru^{3+} complexes in tumour cells might be mediated by transferrin [8].

NADPH oxidase, an enzyme found in phagocytic cells, catalyses the reduction of oxygen to $O_2^{-\bullet}$ using NADPH as the electron donor (see for a review, 17-21). The $O_2^{-\bullet}$ that is formed, along with reactive species derived from it, is used as a potent antimicrobial weapon in the process of host defence. The NADPH oxidase complex consists of two membrane-bound redox centres: a FAD-containing flavoprotein and a heterodimeric *b*-type cytochrome, cytochrome b_{-245} (also called cytochrome b_{558}). The oxidase is dormant in resting neutrophils, but acquires catalytic activity when the cells are exposed to appropriate stimuli [22]. The components of NADPH oxidase exist in the form of a complex that contains the cytosolic proteins $p47^{phox}$ and $p67^{phox}$. The fact that the $O_2^{-\bullet}$-forming NADPH oxidase is always recovered in the particulate fraction suggests that, in the course of physiological enzyme activation, cytosolic components are translocated to the membrane.

The present study was undertaken to analyse whether the novel Ru–PDTA complex was able to activate the respiratory burst in human neutrophils. This activity could be associated with the cytostatic capacity of specific cells, such as neutrophils, which participate in the defence mechanisms. It is known that activated phagocytic cells have the capacity to exert cytotoxicity towards tumour cells *in vitro*. It is also known that phagocytic cells infiltrate tumour nodes and that they are very proximal to tumour cells [23]. The present paper provides evidence that the Ru–PDTA complex stimulates $O_2^{-\bullet}$ production and also that it increases cytoplasmic Ca^{2+} levels by human neutrophils *in vitro*. This effect could mediate the tumour regression that was observed with the Ru–PDTA complex in previous studies [24].

EXPERIMENTAL

Materials

Ru–PDTA complex (molecular mass 548.3) was synthesized as described previously [14]. Chemicals were of analytical grade

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free calcium concentration; DPI, diphenylene iodonium; Ru–PDTA, complex from ruthenium (Ru^{3+}) and propylene-1,2-diaminotetra-acetic acid {PDTA, $[Ru(C_{11}H_{15}N_2O_8)Cl_2]H,4H_2O$}; $O_2^{-\bullet}$, superoxide anion.

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from Merck (Darmstadt, Germany). Cytochrome *c*, Fura-2 acetoxymethyl ester, pertussis toxin, dibutyryl cyclic AMP and horseradish peroxidase conjugated with goat anti-mouse IgG were obtained from Sigma Chemical (Madrid, Spain). Other biochemical reagents were from Boehringer (Mannheim, Germany). 2-Mercaptoethanol, SDS, acrylamide, *NN′*-methylenebisacrylamide, Coomassie Brilliant Blue R-250 and nitrocellulose blotting membranes were purchased from Bio-Rad (Richmond, CA, U.S.A.). Ficoll 400 and molecular-mass standards were from Pharmacia (Uppsala, Sweden). Staurosporine was purchased from Calbiochem–Boehringer (La Jolla, CA, U.S.A.). Rabbit polyclonal antibodies raised against $p47^{phox}$ and $p67^{phox}$, together with diphenylene iodonium (DPI) were kindly given by Professor O. T. G. Jones (Department of Biochemistry, University of Bristol, Bristol, U.K.). Monoclonal antibody specific for phosphotyrosine (PY20) was purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) was purchased from Serva (Spain) and 4-iodophenol was from Aldrich.

Neutrophil preparation and culture

Neutrophils were obtained from normal adult human subjects and purified by dextran sedimentation and Ficol–Hypaque centrifugation as indicated in [25]. Neutrophils (5×10^6 cells/ml) were suspended in Krebs–Ringer bicarbonate buffered with Hepes (KRB), pH 7.4 (118 mM NaCl/4.75 mM KCl/1.18 mM KH_2PO_4/1.18 mM $MgSO_4$/10 mM Hepes/25 mM $NaHCO_3$/1.25 mM $CaCl_2$) and containing 10 mM glucose. Further additions are described in the legends to the individual Figures.

Preparation of cytosolic and membrane fractions

After different times of incubation cells were centrifuged (800 ***g***) and lysed in buffer A [100 mM Hepes (pH 7.3)/100 mM KCl/3 mM NaCl/3 mM $MgCl_2$/1.25 mM EGTA/1 mM PMSF/5 μg/ml aprotinin/156 μg/ml benzamidine]. The cells were disrupted by sonication on ice (20 W for three bursts of 5 s each, separated by 30 s intervals). Unbroken cells and debris were removed by centrifugation at 10000 ***g*** for 5 min at 4 °C. The cytosolic fraction was obtained by ultracentrifugation at 92000 ***g*** for 60 min at 4 °C, as indicated in [26]. The pellets were resuspended in the sonication buffer B, with detergent [120 mM NaH_2PO_4 (pH 7.4)/1 mM $MgCl_2$/1 mM EGTA/1 mM dithiothreitol/20 % glycerol and 40 mM octyl glucoside] and then centrifuged at 18000 rev./min (20000 ***g***) for 40 min at 4 °C. The resulting pellets were used as the membrane fraction.

Measurement of $O_2^{-\cdot}$ release by neutrophils

Neutrophils (0.5×10^6 cells/ml) were added to paired cuvettes containing 80 μM cytochrome *c* in KRB with a final assay volume of 1 ml. $O_2^{-\cdot}$ was measured by monitoring the superoxide dismutase-inhibitable reduction of cytochrome *c* at 550 nm, at 37 °C, using a diode-array double-beam spectrophotometer (Hewlett Packard) [27]. $O_2^{-\cdot}$ production was stimulated by different concentrations of the Ru–PDTA complex or 100 nM mezerein. $O_2^{-\cdot}$ generation was calculated by using a molar absorption coefficient of 21.1 $mM^{-1} \cdot cm^{-1}$.

Electrophoresis, immunoblotting and detection by enhanced chemilumiscence

Membrane and cytosolic fractions were subjected to SDS/PAGE on 10 % polyacrylamide gels using the Laemmli buffer system [28]. Separated proteins were transferred on to a nitrocellulose membrane, which was blocked with 5 % defatted milk. The membrane was incubated with a mixture of partially purified rabbit antibodies raised against $p47^{phox}$ and $p67^{phox}$ and finally detected with horseradish peroxidase conjugated with goat anti-rabbit IgG (1:5000 final dilution). The bound secondary antibody was detected by enhanced chemiluminescence. Briefly, membranes were incubated for 1 min in 10 ml of fresh luminescence reagent solution, composed of 10 mM HCl/Tris, pH 8.5, 2.25 mM luminol, 0.015 % (v/v) H_2O_2 and 0.45 mM 4-iodophenol, the latter acting as enhancer of the chemiluminescence reaction [29]. These concentrations of luminol, H_2O_2 and 4-iodophenol had been determined to be optimal for maximum light production (M. Carballo, G. Marquez and F. Sobrino, unpublished work). Luminol and 4-iodophenol were freshly prepared in 10 ml of 10 mM Tris/HCl, pH 8.5. Luminol was previously dissolved in 50 μl of 1 M NaOH. The use of DMSO as solvent should be avoided since, under alkaline-DMSO conditions, luminol autoxidizes with emission of intense luminiscence [30]. After 1 min of incubation the membranes were placed on a filter paper, covered with Saran wrap ('clingfilm') and exposed to X-ray films (X-OMAT; Kodak) in the dark for 1–5 min.

Analysis of protein tyrosine phosphorylation

Neutrophils (5×10^6 cells) in 0.5 ml of KRB were pretreated with different concentrations of the Ru–PDTA complex. The reaction was terminated at 20 min by centrifugation and aspiration of the reaction medium. A volume (75 μl) of sample buffer [28] containing 125 mM Tris/HCl (pH 6.8)/2 % SDS/5 % glycerol/5 % mercaptoethanol/0.003 % Bromophenol Blue was added to the pellet, and the samples were boiled for 5 min. The cell lysates were resolved by SDS/10 %-PAGE. Separated proteins were electrotransferred to a nitrocellulose membrane. After blocking with 3 % BSA/1 mM PMSF/5 μg/ml aprotinin in TBST [150 mM NaCl/50 mM Tris/HCl (pH 7.5)/0.1 % Tween 20] the membrane was incubated with anti-phosphotyrosine antibody (PY20) (1:2000 final dilution). Bound antibodies were detected by luminol enhanced chemiluminescence, as indicated above. Protein concentrations were analysed as previously described [31].

Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} was analysed as described in [25]. Briefly, neutrophils were incubated in a medium (Ca^{2+} medium) composed of 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl, 1 mM $CaCl_2$, 20 mM Hepes at pH 7.4, at a concentration of 10^7 cells/ml at 37 °C for 45 min in the presence of 2 μM Fura-2 acetoxymethyl ester and 2.5 mM probenecid. The excess of the dye was then washed away, and the neutrophils were kept in the same medium. Batches of 10^7 cells were resuspended in 0.6 ml of Ca^{2+} medium at 37 °C, and the Ru–PDTA complex was added to the cellular suspension. Fura-2 fluorescence ($\lambda_{excitation}$ at 340 nm and $\lambda_{emission}$ at 500 nm) was recorded on a Perkin–Elmer LS-5 spectrofluorimeter. Ca^{2+} was calibrated using a value of 224 for the K_d of Fura-2.

RESULTS

Stimulation of the respiratory burst by the Ru–PDTA complex

The tumour promoter PMA has been described as the most active stimulator of the respiratory burst [32]. In preliminary experiments, human neutrophils were incubated with PMA and the Ru–PDTA complex. It was found that the complex did not alter a PMA-dependent respiratory burst (results not shown).

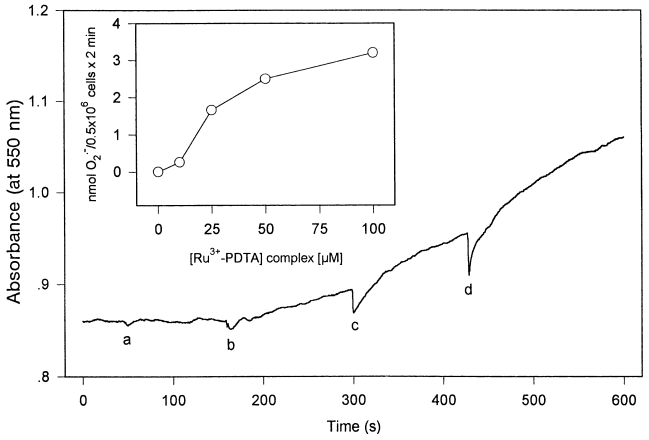

Figure 1 Absorption measurement showing the reduction of cytochrome *c* after neutrophil activation by the Ru–PDTA complex

Neutrophils (0.5×10^6 cells/ml) were incubated with 10 μM (**a**), 25 μM (**b**), 50 μM (**c**) and 100 μM (**d**) for the indicated times. Data are representative of three independent experiments. The dose-dependence of $O_2^{-\cdot}$ ($= O_2^-$) production is illustrated in the inset.

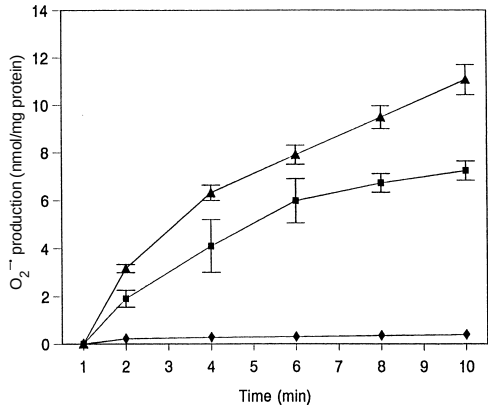

Figure 3 Effect of DPI and staurosporine on the Ru–PDTA-complex-induced ($O_2^{-\cdot}$) production by neutrophils

Neutrophils (0.5×10^6 cells/ml) were incubated with 100 μM Ru–PDTA complex in the absence (▲) or in the presence of 10 μM DPI (■) or 1 μM staurosporine (◆) for 10 min at 37 °C. Data are means ± S.E.M. for three independent experiments performed in duplicate.

However, when the Ru–PDTA complex was added alone to cell suspensions, a clear production of $O_2^{-\cdot}$ was found (Figure 1). Successive additions of the Ru–PDTA induced a rapid stimulation of $O_2^{-\cdot}$ production by neutrophils which seemed to be sustained after 10 min of incubation. This $O_2^{-\cdot}$ production was dependent upon the Ru–PDTA complex concentration with an apparent $k_{0.5}$ of approx. 25 μM. (Figure 1, insert). It was verified that some preparations of Ru–PDTA complex, in the absence of cells, produced a slight decrease in cytochrome *c*, which oscillates between 8 and 18 % of the total change of absorbance, measured when Ru–PDTA complex was added to cells. That unspecific change of absorbance was substracted from experimental values to obtain the specific Ru–PDTA-dependent $O_2^{-\cdot}$ production.

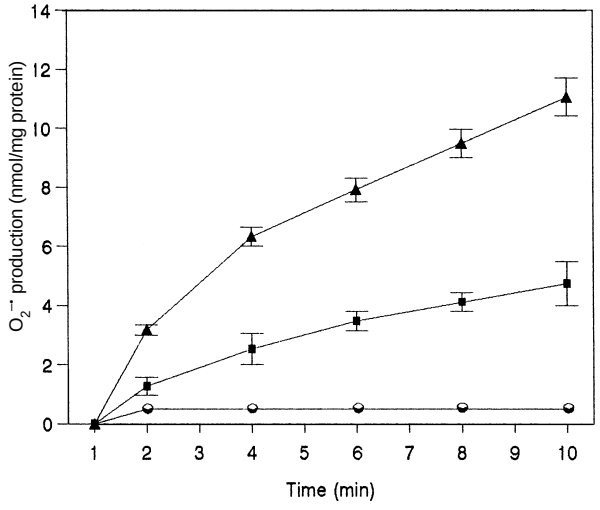

Figure 2 Kinetics of production stimulated by the Ru–PDTA complex, $RuCl_3$ and PDTA by human neutrophils

Neutrophils (0.5×10^6 cells/ml) were incubated with 25 μM (■) or 50 μM (▲) of the Ru–PDTA complex, and 25 μM $RuCl_3$ (○) or 25 μM PDTA (●). Values are means ± S.E.M. for three independent experiments.

Also, when Ru–PDTA complex was added to inactivated cells (e.g. by heat), it had not effect. Further experiments were then designed to assess whether the stimulatory effect of the Ru–PDTA complex was due to the Ru–PDTA molecule itself or was due to one or more of its constituents (e.g. Ru_3Cl, PDTA). Figure 2 shows that only the Ru–PDTA complex had the capacity to stimulate $O_2^{-\cdot}$ production, since neither $RuCl_3$ nor PDTA at 25 μM had any effect. Concentrations of the constituents higher than 25 μM had no effect (results not shown). We then analysed the specifity of Ru–PDTA-dependent $O_2^{-\cdot}$ production. Figure 3 illustrates that diphenylene iodonium (DPI), a well-known inhibitor of NADPH oxidase [33], decreased $O_2^{-\cdot}$ production by approx. 25 %. DPI at doses higher than 10 μM produced further inhibition, but this never reached 100 %. Previous data with protein kinase inhibitors have demonstrated that NADPH oxidase activation is mediated by protein phosphorylation [34,35]. The presence of 1 μM staurosporine produced a complete inhibiton of Ru–PDTA-dependent formation $O_2^{-\cdot}$ (Figure 3). The lower inhibition observed with DPI may be interpreted in terms of previous findings, which indicate the necessity of reducing conditions for inhibition of $O_2^{-\cdot}$ production by DPI [36]. Since the Ru–PDTA complex forms an oxidant environment [37], this may explain the decreased inhibitory capacity of DPI. The response to the Ru–PDTA complex was not altered by pertussis-toxin pretreatment, indicating that the involvement of G_i-type guanine-nucleotide-regulatory protein was unlikely. Since pertussis toxin produces an activation of adenyl cyclase, the effect of dibutyryl cyclic AMP was also investigated (results not shown). In agreement with the observations with pertussis toxin, cyclic AMP did not alter the response to the Ru–PDTA complex. Previously [38] it was shown that cyclic AMP or agents that elevate intracellular levels of cyclic AMP decrease $O_2^{-\cdot}$ production [38]. However, it has been shown recently that cyclic AMP treatment does not alter PMA-dependent $O_2^{-\cdot}$ production in neutrophil-like HL-60 cells [39].

Translocation of NADPH oxidase subunits and phosphorylation on tyrosine residues induced by the Ru–PDTA complex

Studies performed with neutrophils treated PMA and other stimuli have shown that translocation of cytosolic $p47^{phox}$ and $p67^{phox}$ to the plasma membrane is an essential process for the

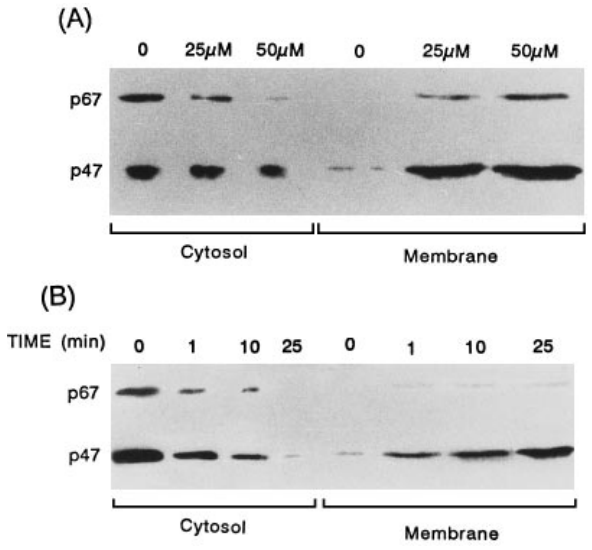

Figure 4 Effect of the Ru–PDTA complex on the translocation of p47^{phox} (p47) and p67^{phox} (p67) from cytosol to the membrane

(**A**) Neutrophils (5×10^6 cells/ml) were incubated with 25 and 50 μM Ru–PDTA complex for 20 min at 37 °C. Cytosolic and membrane fractions were separated and electrotransferred as indicated in the Experimental section. The nitrocellulose membrane was then probed with anti-p47^{phox} and anti-p67^{phox} antisera and proteins were revealed with luminol-enhanced chemiluminiscence. (**B**) Neutrophils were exposed to the Ru–PDTA (50 μM) for the indicated times, and then analysed for p47^{phox} and p67^{phox} as indicated in the Experimental section. This Figure is representative of three independent experiments.

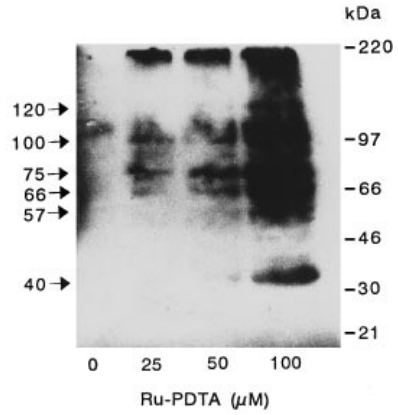

Figure 5 Tyrosine phosphorylation induced by the Ru–PDTA complex and analysed by anti-phosphotyrosine immunoblotting

Neutrophils (5×10^6 cells/ml) were pretreated for 20 min without complex (0 μM) or in the presence of 25, 50 or 100 μM of Ru–PDTA complex. Phosphotyrosine was detected using a luminol-enhanced chemiluminescence method. Arrows indicate molecular masses calculated from the positions of the molecular-mass standards. This Figure is representative of three independent experiments.

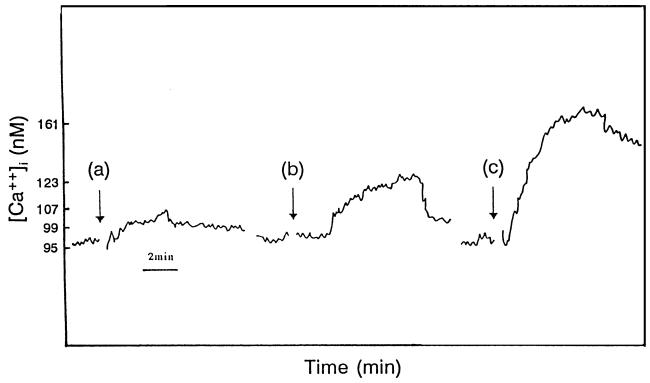

Figure 6 Dose-dependent effect of the Ru–PDTA on cytosolic free Ca^{2+} (Ca^{++})

The Ru–PDTA complex was added as follows: 2 μM (a), 5 μM (b) and 25 μM (c). The mean change in peak value of $[Ca^{2+}]_i$ at 25 μM of Ru–PDTA complex was (means $\pm$ S.E.M., $n = 3$) 164 ± 6 nM. The Ca^{2+} concentration in resting cells was 91 ± 4 nM. The results shown are representative of three experiments.

activation of the NADPH oxidase ([40–42]; for a review, see [19]). In order to compare the translocation from cytosol to the membrane of cytosolic proteins p47^{phox} and p67^{phox}, cytosolic and membrane fractions from resting neutrophils or those stimulated with the Ru–PDTA complex were subjected to electrophoresis and immunoblotted with specific anti-p47^{phox} and anti-p67^{phox} polyclonal antibodies. The results are shown in Figures 4(A) and 4(B). Figure 4(A) shows that, with 25 μM of the Ru–PDTA complex, there was a clear translocation of both proteins from the cytosol to the plasma membrane after 20 min and that this translocation was more pronounced with 50 μM Ru–PDTA. In our hands p47^{phox} was always readily detected using the polyclonal antisera. By contrast, detection of p67^{phox}, particularly in plasma-membrane preparations, was less reliable. This could be explained by the recent finding of Heyworth et al. [43] that p67^{phox} is located in the Triton X-100-insoluble fraction. The kinetics of the translocation are shown in Figure 4(B). At 1 min after treatment with the Ru–PDTA complex, p47^{phox} was clearly detectable in the plasma membrane, whereas in cytosolic fractions the opposite was observed. This translocation seemed to be completed after 25 min, since no cytosolic p47^{phox} was detected at this time. The p67^{phox} protein showed similar kinetics of disappearance from the cytosol, but was barely detectable in the plasma-membrane fraction.

It is well established that activation of NADPH oxidase by different stimulants is accomplished by tyrosine phosphorylation of several cytosolic proteins [44–46]. The next experiments were therefore designed to assess whether the Ru–PDTA complex produced a similar effect. Figure 5 shows that the Ru–PDTA complex induced tyrosine phosphorylation of 40, 57, 66, 75, 100, and 120 kDa proteins in a dose-dependent manner. It is noteworthy that, in these last-mentioned experiments, the cytosolic and membrane fractions were processed together. A similar pattern of phosphorylation was previously observed in neutrophils incubated with different stimuli [43–46]. When cells were stimulated with Ru–PDTA complex in the presence of staurosporine (1 μM), a diminished protein phosphorylation was detected (results not shown). This fact agrees with previous observations in human neutrophils [34].

Effect of the Ru–PDTA complex on Ca^{2+} levels

Ca^{2+} appears to play a role as a second messenger in the neutrophil activation sequence, just as it does in other systems, and the relative roles of intra- and extra-cellular calcium have been assessed [47]. A rapid increase in cytosolic Ca^{2+} has been demonstrated in neutrophils in response to a variety of stimuli [48]. Figure 6 shows that the Ru–PDTA complex increased intracellular Ca^{2+} in a dose-dependent manner and, at 5 μM Ru–

PDTA complex, a clear effect was detectable. At 25 μM Ru–PDTA complex a rapid onset of intracellular Ca^{2+} levels was detected. This observation suggests a relationship between the increased Ca^{2+} levels and the activation of the oxidase. However, this classical Ca^{2+}-dependent signalling pathway has been discussed in terms of different data (see [19] for a review), which have demonstrated the possibility, under appropiate circumstances, of a Ca^{2+}-independent oxidase activation. Also the effect of Ru–PDTA was analysed in the presence of staurosporine (1 μM). In this case staurosporine had no effect (results not shown). The results obtained with staurosporine on Ca^{2+} levels and phosphorylation on protein provided further evidence that different signal-transduction processes are involved in Ru–PDTA-dependent neutrophil activation.

In order to study the specific effect of Ru–PDTA complex on neutrophils, other non-phagocytic cells (e.g. blood T-cell lymphocytes) were analysed. In the presence of Ru–PDTA complex, no production of $O_2^{-\bullet}$ was detected in these cells. However, Ru–PDTA complex was able to increase intracellular Ca^{2+} and phosphorylation of proteins (results not shown), as it did in neutrophils.

DISCUSSION

We have presented evidence that a novel complex, derived from Ru^{3+} and a chelanting agent, PDTA, was able to stimulate $O_2^{-\bullet}$ production by human neutrophils. This stimulation was specific for the complex, since the individual constituents had no effect. Several lines of evidence suggest that the complex acts as a true stimulator of NADPH oxidase. First, the mechanism of activation seems to be associated with the translocation of cytosolic proteins, $p47^{phox}$ and $p67^{phox}$, from cytosol to the plasma membrane, since the assembly of the NADPH oxidase components is a well-established phenomenon for other defined stimulators of the enzyme [40–42]. The critical functional importance of these two proteins was established by studies of a subset of chronic-granulomatous-disease patients, who exhibit autosomal inheritance, normal membrane content of cytochrome b_{558} and a defect localized to the cytosol in a cell-free oxidase system. Secondly, the overall inhibition by staurosporine of the Ru–PDTA-complex-dependent activation of NADPH oxidase suggests the participation of protein kinase C, in a manner similar to other stimuli. Also, a partial unresponsiveness of the Ru–PDTA complex to DPI inhibition was detected, which could be due to the requirement for a reduced environment for DPI activity, as suggested by other authors [36]. Thirdly, the Ru–PDTA complex elicited phosphorylation at tyrosine residues on several proteins (Figure 5), with a phosphorylation pattern very similar to that described previously [43–46]. In addition, the short time required to produce a measurable response to the Ru–PDTA (Figure 1) suggests that non-specific or cytotoxic effects are unlikely. The stimulation of the respiratory burst elicited by the Ru–PDTA complex seems to be independent of cyclic AMP levels, since neither dibutyryl cyclic AMP nor pertussis toxin (which increases cyclic AMP levels) altered the $O_2^{-\bullet}$ production.

Our present findings on the stimulation of the respiratory burst by the Ru–PDTA complex provides a hypothetical model to explain the antitumour properties of the compound. Evidence based on structural studies indicates that phagocytic cells infiltrate tumour nodes, and this defence mechanism has been observed in different tumour types [23,49]. Conversely, the administration of agents that induce macrophage disruption or dysfunction has been reported to increase host susceptibility to neoplasia [50]. Also tumour rejection elicited by interleukin-4 [23] or interleukin-13 [49], delivered to the site of the tumour, is the result of direct membrane and cytoplasmic damage to tumour cells by eosinophils, neutrophils and macrophages that deeply penetrate the proliferating tumour mass. Thus the death of tumour cells elicited by the Ru–PDTA complex could be interpreted as having been caused by the infiltrating neutrophils (and other phagocytic cells). These cells, located near to tumour nodes, are activated the by the Ru–PDTA complex to produce an increased level of free radicals (e.g. $O_2^{-\bullet}$), thereby providing damage in the proximal tumour cells. Previously it has been shown that another antineoplasic agent, a muramyl dipeptide analogue, enhances killing activity on tumour cells and the synthesis of $O_2^{-\bullet}$ by neutrophils [51]. Although the $O_2^{-\bullet}$ and other reactive oxygen intermediates have traditionally been viewed primarily as potent microbicidal agents synthesized by phagocytic cells [52], the complexity of the phenomenon it is now well recognized. The specifity of an oxygen-free radical-mediated response is likely to depend on the activation status of individual cells, as well as interference by, and integration with, other possible concomitant signalling events [53].

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