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Cellular localization of antiviral polyoxometalates in J774 macrophages

Lan Ni[a], Phillip Greenspan[b], Robert Gutman[b], Cathy Kelloes[c], Mark A. Farmer[c], F. Douglas Boudinot[a,*]

[a]*Department of Pharmaceutics, College of Pharmacy, University of Georgia, Athens, Georgia 30602-2353, USA*
[b]*Department of Pharmacology and Toxicology, College of Pharmacy, University of Georgia, Athens, Georgia 30602, USA*
[c]*Center for Advanced Electron Microscopy, University of Georgia, Athens, Georgia 30602, USA*

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Abstract

The cellular localization of the polyoxometalates, $K_{12}H_2[P_2W_{12}O_{48}]\cdot 24H_2O$ (JM 1591), $K_{10}[P_2W_{18}-Zn_4(H_2O)_2O_{68}]\cdot 20H_2O$ (JM 1596), and $[Me_3NH]_8[Si_2W_{18}Nb_6O_{77}]$ (JM 2820) were examined in cultured J774 cells by inhibition of cellular uptake of acetylated low-density lipoprotein (LDL) and by electron microscopy. All three polyoxometalates inhibited the cellular uptake of acetylated LDL, suggesting that the polyoxometalates block the association of acetylated LDL with cellular scavenger receptors. Fluorescence microscopy showed increased numbers of vacuoles in the presence of polyoxometalates, suggesting their uptake by cells. Using scanning electron microscopy (SEM), no significant cell surface morphological differences were observed between treated and non-treated J774 cells, suggesting that the compounds are not toxic to J774 cells up to a concentration of 200 μg/ml. Transmission electron microscopy (TEM) revealed large amounts of high electron dense granules were observed in the ramifying system of tubular cavities and vacuoles. TEM–energy dispersive spectroscopy (EDS) X-ray microanalysis was unable to differentiate the dense particles, most likely because the amount of tungsten in the cells was below the limit of detection. X-ray microanalysis conducted using a SEM–wavelength dispersive spectroscopy (WDS) detected tungsten, averaging $0.45 \pm 0.16\%$ (mean $\pm$ S.D.), in the J774 cells treated with JM 2820, suggesting that this polyoxometalate was taken up by the macrophages or was bound to their surface. Polyoxometalates interact at the cell surface and appear to be taken up by J774 macrophages. The cellular localization of polyoxometalates may be associated with anti-HIV activity.

Keywords: Polyoxometalates; J774 cells; Macrophage; Electron microscopy

* Corresponding author. Tel: +706 5425335; fax: +706 5425346; e-mail: boudinot@rx.uga.edu

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1. Introduction

Polyoxometalates are aggregates of condensed oligomeric polyanions of oxide ions and early transition-metal cations with a high density of negative charge. The polyoxometalates JM 1591 ($K_{12}H_2[P_2W_{12}O_{48}]\cdot 24H_2O$) (Contant and Ciabrini, 1977), JM 1596 ($K_{10}[P_2W_{18}Zn_4(H_2O)_2O_{68}]\cdot 20H_2O$) (Finke and Droege, 1984), and JM 2820 ($[Me_3NH]_8[Si_2W_{18}Nb_6O_{77}]$) (Finke et al., 1987) exhibit potent antiviral activities against a variety of viruses including human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV- 2) in vitro. Previous pharmacokinetic studies in rats (Ni et al., 1994) demonstrated that, after intravenous administration, these polyoxometalates are widely distributed throughout the body. The compounds accumulated in various tissues with the highest concentrations detected in kidney and liver. Further, the renal and biliary clearances of the compounds were non-linear (Ni and Boudinot, 1995). For each polyoxometalate, systemic clearance was higher than the combined renal and biliary clearance assessed by determining excreted tungsten, suggesting that the polyoxometalates may irreversibly distribute intracellularly in tissues. Indeed, high amounts of the compounds, up to 34% of the administered dose, were found in tissues 1 week following single dose intravenous administration.

Although the mechanism of anti-HIV activity of polyoxometalates has not been fully elucidated, two mechanisms including inhibition of reverse transcriptase (RT) and inhibition of binding/fusion process, are considered to be responsible for their antiviral activity (Hill et al., 1990). Other mechanisms may also be involved. The mechanisms of action of polyoxometalates appear to be associated with their binding to the cell surface or with the ability of the compounds to enter cells. The determination of the cellular localization of polyoxometalates may provide insight into their mechanisms of action.

Macrophages are responsible for the removal of a range of polyanionic macromolecular ligands including modified albumin and acetylated low density lipoprotein (LDL) (Fraser and Gordon, 1993). The macrophage scavenger receptors mediate this endocytosis process, and acetylated LDL and polyanions are competing ligands for scavenger receptors. J774 cells are a murine macrophage cell line with properties similar to other macrophages (Defendi, 1976). This cell line is often used to examine the uptake of polyanionic compounds and modified LDL in relation to the formation of macrophage foam cells (Goldstein et al., 1979; Lee et al., 1992). To investigate the possible mechanisms of anti-HIV activity of polyoxometalates, the in vitro cellular uptake and localization of the compounds were examined in the J774 macrophage cell line.

2. Materials and methods

2.1. Materials

JM 1591, JM 1596, and JM 2820 were provided by the Biomedical Research Division of Johnson Matthey (West Chester, PA, USA). HPA-23 ($(NH_4)_{17}(H)[NaSb_9W_{21}O_{86}]$) was provided by Dr Raymond Schinazi (Emory University/VA Medical Center, Atlanta, GA, USA). Epoxide resin for embedding cells for electron microscopy studies contained 28.2% dodecyl succinic anhydride (DDSA), 45.4% Epon 812, 25.2% methyl nadic anhydride (MNA), and 1.2% 2,4,6-tridimethylaminomethyl phenol (DMP-30). These chemicals, as well as glutaraldehyde, osmium tetroxide (OsO_4), propylene oxide and ethyl alcohol, were obtained from Polysciences (Warrington, PA, USA).

2.2. J774 cells

J774 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Culture medium, as well as supplements, were purchased from Whittaker M.A. Bioproducts (Walkersville, MD, USA).

2.3. Inhibition of J774 cell uptake of acetylated LDL by polyoxometalates

To examine the possible role of macrophage scavenger receptors in mediating the cellular

uptake of polyoxometalates, the inhibition of the uptake of acetylated LDL by polyoxometalates was investigated. The uptake of ^{125}I-acetylated LDL was determined as described (Goldstein and Brown, 1974). Briefly, in 35-mm tissue culture dishes, macrophages were incubated in fresh Dulbecco's modified Eagle medium (DMEM) with 10% heat inactivated fetal calf serum (FCS) containing ^{125}I-acetyl-LDL (5 μg of protein/ml) and JM 1591, JM 1596, JM 2820 or HPA-23 (15 μg/ml). After incubation at 37°C for 4 h, the dishes were washed four times with 1.5 ml phosphate buffered saline (PBS) containing 2 mg/ml bovine serum albumin (BSA), after which they were washed four times with 2 ml PBS. 1 ml of 0.2 M NaOH was added to each dish for 1 h, and the cell digest was collected. The amount of internalized ^{125}I-acetyl-LDL was determined by counting 0.5 ml of the cell digest in a gamma counter (Beckman G 5500). Protein concentration was determined by the Lowry method (Lowry et al., 1951). The culture medium (0.5 ml) was treated with 1.5 ml 20% cooled trichloroacetic acid to precipitate proteins and, after centrifugation (2400 rpm for 20 min), 1 ml supernatant was treated with 10 μl 40% KI and 40 μl 30% H_2O_2. After standing at room temperature for 5–10 min, the iodine was extracted with 2 ml $CHCl_3$. After centrifugation (2400 rpm for 5 min at 4°C), 0.5 ml aqueous phase was counted in the gamma counter to determine the acid soluble degradation product of acetyl-LDL. The amount of internalized and degraded ^{125}I-acetyl-LDL, represents the total amount of the cellular uptake of ^{125}I-acetylated LDL. The percent inhibition of the endocytosis of acetylated LDL by polyoxometalates was determined by comparing the total amount of cellular uptake of ^{125}I-acetylated LDL in polyoxometalate-treated cells and control cells. Experiments were done in triplicate.

2.4. Fluorescence microscopy

Macrophages were incubated with the polyoxometalates (15 μg/ml) as described above at 37°C for 24 h. The cells were then rinsed five times with 2 ml PBS containing BSA, then 3 times with 2 ml PBS. The cells were treated with 1% glutaraldehyde in PBS, stained with nile red (Brown et al., 1992) and observed using fluorescence microscopy (Zeiss Universal Microscope, Carl Zeiss, Thornwood, NJ, USA).

2.5. Scanning electron microscopy (SEM)

Aliquots of J774 cell suspensions were spread on glass coverslips and incubated at 37°C for 4 h to allow cell attachment. Cells on coverslips were incubated with either JM 1591, JM 1596 or JM 2820 at a concentration of 200 μg/ml in culture medium at 37°C for 48 h. The cells of other coverslips were cultured with medium alone and served as controls. After incubation, the cells were washed with PBS three times; fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer for 15 min; rinsed with two changes of 0.1 M cacodylate buffer with 5% sucrose; and dehydrated through 30, 50, 70, 85, 95% ethyl alcohol and two changes of 100% ethyl alcohol (changes were at 10-min intervals). Finally, critical point drying (CPD) was performed for the final sample preparation (SAMPRI 780A). The cell samples were coated with gold/palladium and the morphology of J774 cells were examined by SEM (Philips SEM 505) at 20.0 keV.

2.6. Transmission electron microscopy (TEM)

J774 cells were incubated with and without JM 2820 at a concentration of 50 μg/ml in culture medium at 37°C for 48 h in duplicate. After incubation, cells were washed three times with PBS and the cell suspension was centrifuged at 2000 rpm for 15 min to form a pellet. Cells were resuspended, fixed by adding 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature, and pelleted at 2000 rpm for 10 min. The cell pellet was washed twice with 4 ml of 0.2 M sucrose in 0.1 M cacodylate buffer (pH 7.2–7.4) and stored at 4°C for 48 h. The pellet was resuspended in 2% osmium tetroxide (OsO_4) for 1 h at room temperature to postfix the cells. The cell pellet was reformed by centrifugation (2000 rpm for 10 min) and then rapidly washed

twice in 3 ml distilled water. At 10-min intervals, cells were dehydrated through a graded series of ethyl alcohol solutions: 30, 50, 70, 80 and 95%. Cells were then dehydrated in two changes of 100% alcohol for 20 min in each change. The 100% alcohol was replaced with propylene oxide (PO) for 20 min, and fresh PO was added for another 20 min. Epoxy resin was gradually added in increasing amounts so that the PO/epoxy resin ratio increased from 2:1 to 1:1 to 1:2. Cells remained for 30 min at each PO/epoxy resin ratio. During the procedure, the vial was placed on a slow rotor to enhance infiltration. Following infiltration, cells were pelleted by centrifugation and resuspended in 100% epoxy resin and kept on the rotor overnight. The cell was pelleted again, transferred to BEEM capsules containing fresh 100% epoxy resin. The cell pellet was allowed to settled for 1 h before polymerizing at 60°C overnight. The cell block was thin sectioned with a glass knife using a Sorvall MT-2 ultramicrotome. Sections with 70–100 nm thickness were picked up on 200-mesh copper grids, stained with 2% uranyl acetate for 30 min and rinsed three times in distilled water. The sections were stained in lead citrate for 5 min followed by one rinse in 0.2 M NaOH and three rinses in distilled water. The ultrastructure of the J774 cells treated with or without JM 2820 was examined by using a JEOL JEM 100CX II transmission electron microscope at 80 keV.

2.7. TEM and energy dispersive X-ray microanalysis

Cell suspensions were fixed with or without osmium postfixation, followed by dehydration and embedding as described above for TEM. Thin sections (190–220 nm thick) were cut and placed on either 200-mesh copper grids, 200-mesh nickel grids, 100-mesh nickel grids with Formvar carbon-coated supporting film, or Formvar coated copper slot grids without further contrasting. These sections were then examined on a Philips 400 TEM for morphological detail at 80 keV accelerating voltage. Energy dispersive spectroscopy (EDS) X-ray microanalysis was performed with a NORAN TN 5500 EDS System. Spectra were counted and collected for 600 s over an energy range of 0–20 keV with 100 keV accelerating voltage.

2.8. SEM-probe and wavelength dispersive X-ray microanalysis

Macrophages were incubated with either JM 1591, JM 1596 or JM 2820 at a concentration of 200 μg/ml in DMEM at 37°C for 48 h as previously described. After incubation, cells were washed three times with PBS. Cells were smeared on a glass slide and allowed to air dry. The cells were coated with carbon and examined with a JEOL JXA-8600 Superprobe at 15.0 keV accelerating voltage and 15.2 nA filament current with a take-off angle of 40%. X-ray microanalysis was performed and spectra counted for 600 s over an energy range of 0–10 keV. The Proza correction was then performed.

3. Results

3.1. Inhibition of J774 cell uptake of acetylated LDL by polyoxometalates

The inhibition of J774 cell uptake of acetylated LDL by JM 1591, JM 1596, JM 2820 and HPA-23 is presented in Table 1. Each polyoxometalate, at a concentration of 15 μg/ml, inhibited the cellular uptake of acetylated LDL by approximately 50%. When macrophages incubated with 15 μg/ml polyoxometalate were viewed under the fluorescence microscope, more vacuoles were observed in the nile-red-stained macrophages treated with polyoxometalates than in the control group (data not shown). These results suggest that polyoxometalates are taken up by cells and this possibility was further examined by electron microscopic analysis.

3.2. Morphology of J774 cells under SEM

Scanning electron micrographs of J774 cells treated with and without JM 1591, JM 1596 and JM 2820 are shown in Fig. 1. Some cells appear to be newly attached to the glass surface, while

Table 1
Inhibition of the endocytosis of acetylated LDL in J774 macrophages by polyoxometalates (15 μg/ml)

	Control	JM 1591	JM 1596	JM 2820	HPA-23
Internalization [a]	0.95 ± 0.10 [d]	0.64 ± 0.04	0.60 ± 0.06	0.56 ± 0.06	0.64 ± 0.08
Degradation [b]	1.26 ± 0.10	0.50 ± 0.07	0.50 ± 0.08	0.55 ± 0.09	0.44 ± 0.05
% Inhibition [c]		48.4	49.8	49.8	51.1

[a] Amount of internalized ^{125}I-acetyl-LDL, expressed as μg acetyl-LDL/mg cell protein.
[b] Amount of the acid soluble degradation product of ^{125}I-acetyl-LDL, expressed as μg acetyl-LDL/mg cell protein. The amount of internalized and degraded ^{125}I-acetyl-LDL represents the total amount of cellular uptake of ^{125}I-acetylated LDL.
[c] Inhibition of the total amount of cellular uptake of ^{125}I-acetylated LDL by polyoxometalates relative to the control.
[d] Values are mean ± S.D., $n = 3$.

other cells are firmly attached and flattened out. Numerous complex ridges or folds representing ruffles can be seen on the cell surfaces. The cells which spread more on the surface also show surface folds or ruffles in the central part of the cell and a peripheral skirt of cytoplasm with projecting, flattened processes. Pseudopodia with fine filopodia and larger finger-like processes are also evident. There were no observed morphological differences between polyoxometalate-treated and non-treated J774 cells (Fig. 1).

3.3. Ultrastructure of J774 cells under TEM

Transmission electron micrographs of J774 macrophages treated with and without JM 2820 are shown in Fig. 2. The ultrastructure of the control J774 macrophages is circular, or nearly so, in outline. The cell surface, however, is irregular with pseudopodia projecting from it. Some of these are clearly finger-like, showing a circular cross-section while others are flap-like. The larger of these pseudopodia are flattened back against the surface of the cell and enclose cleft-like spaces. The membrane systems can be observed clearly in the macrophage cytoplasm. Some tubular cavities or lumen are observed. The granular endoplasmic reticulum formed by the lamina and ribosomes are well developed. Many electron dense bodies, present in the cytoplasm, are probably lysosomes (Herscowitz et al., 1981). The mitochondria of the macrophages are elongated with the usual double membrane and shelf-like cristae. The nuclear structure is well demonstrated: nucleopores are prominent, the lamina below the double nuclear membrane is well formed and the chromatin shows related perichromatin and interchromatin granules.

In contrast to the control cell, TEM of thin sections of embedded, JM 2820-treated cells reveals a significant presence of large amounts of electron dense granule deposits in the ramifying system of tubular cavities. High dense granules are also observed in the vacuoles. The cell surface is irregular showing numerous cytoplasmic processes. More vacuoles are observed in the cytoplasm. The absence of breaks or other defects in the plasma membrane and the presence of electron dense granules within the cell suggest that JM 2820 is endocytosed by the macrophages.

3.4. TEM–EDS and SEM–WDS X-ray microanalysis

To obtain X-ray spectra from regions of the cytoplasm containing the high electron dense particles, TEM–EDS X-ray microanalysis was performed. However, the results were inconclusive, probably due to the low concentrations of tungsten and low resolution of the tungsten X-ray peak with other atoms including osmium, copper and nickel by EDS.

To examine the possibility of detecting the cellular content of tungsten in polyoxometalates, microprobe WDS X-ray microanalysis was performed. A tungsten-positive spectrum was obtained from cells treated with JM 2820 (Fig. 3). While the peak appears to be barely above background, approximately 0.45 ± 0.16% (mean ± S.D.; $n = 3$) of atoms were identified as tungsten.

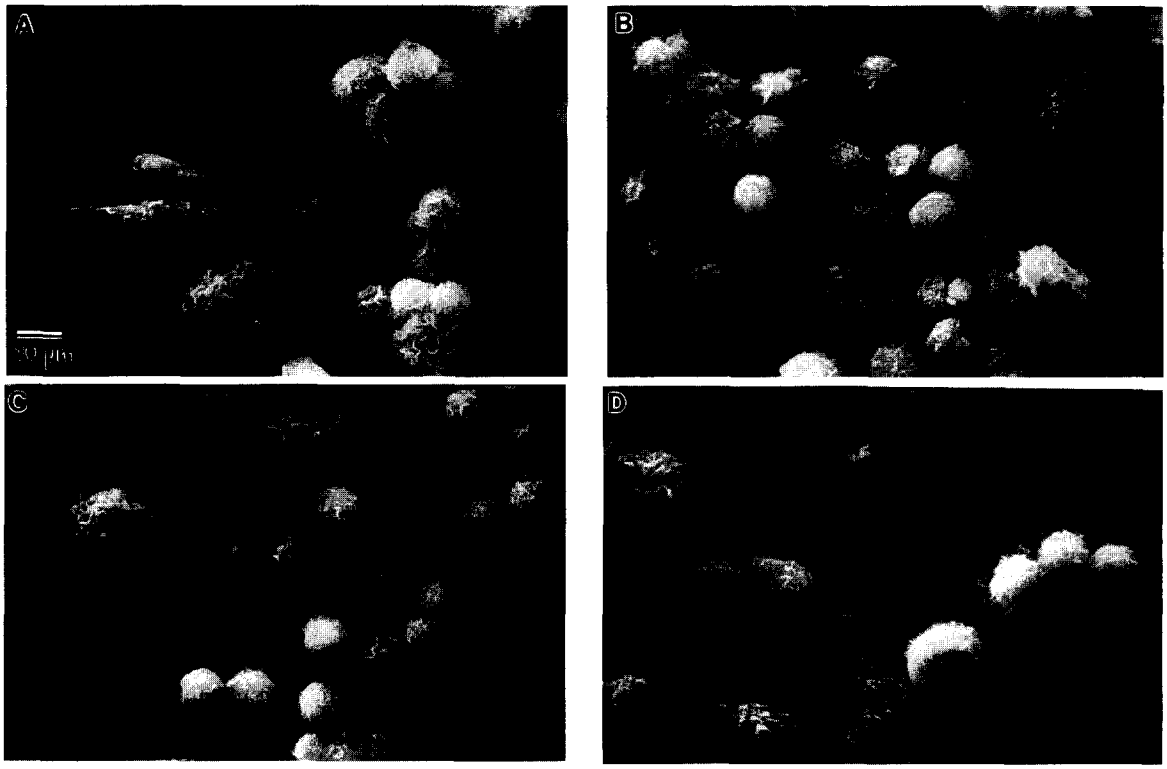

Fig. 1. Scanning electron micrograph (SEM) of J774 macrophages from (A) control, and cells incubated with (B) JM 1591, (C) JM 1596 and (D) JM 2820, at a concentration of 200 μg/ml for 48 h. Scale bar represents 10 μm for all groups.

However, J774 macrophages treated with JM 1591 and JM 1596 showed no evidence of tungsten accumulation.

4. Discussion

It is well known that macrophages are responsible for the removal of a range of polyanionic macromolecular ligands including modified albumin and acetylated LDL (Fraser and Gordon, 1993). The macrophage scavenger receptors mediate this endocytosis process, and acetylated LDL and polyanions are competing ligands for scavenger receptors. Polyoxometalates are aggregates of condensed oligomeric polyanions of oxide ions and early transition-metal cations with a high density of negative charge. The molecular mass of the polyoxometalates are 3940 Da for JM 1591, 5148 Da for JM 1596, 5635 Da for JM 2820 and 6917 Da for HPA-23. In this study, polyoxometalates inhibited 50% of acetylated LDL uptake by J774 cells. These results suggest that both polyoxometalates and acetylated LDL bind to the scavenger receptor, and may be taken up by endocytosis into the cell. It was previously reported that polyoxometalates potently inhibit the binding or fusion of chronically-infected HIV lymphocytes with healthy uninfected cells (Hill et al., 1990). This inhibition was competitive and associated with size, charge density, and other properties of the compounds.

The high electron dense particles observed in the cytoplasm of JM 2820-treated J774 cells under the TEM resemble those seen in Kupffer cells from mice given intravenous thorotrast (Carr, 1973). This heavy metal was also present in vacuoles and vesicles within the Kupffer cell. When

C3HBi fibroblasts were cultured with HPA-23, a similar image was seen (Cibert and Jasmin, 1982). These high dense particles were identified as HPA-23 native crystal form by X-ray fluorescence spectroscopy. In the present study, TEM–EDS X-ray microanalysis was not able to identify the high electron dense granules in the cytoplasm; however, the significantly different ultrastructure of the control and treated J774 cells suggest that JM 2820 is taken up by macrophages and concentrated in the cytoplasm.

X-ray microanalysis was employed to identify the tungsten atoms associated with the cell. TEM–EDS X-ray microanalysis may have failed to identify the high electron dense granules in the cytoplasm because the amount of tungsten present

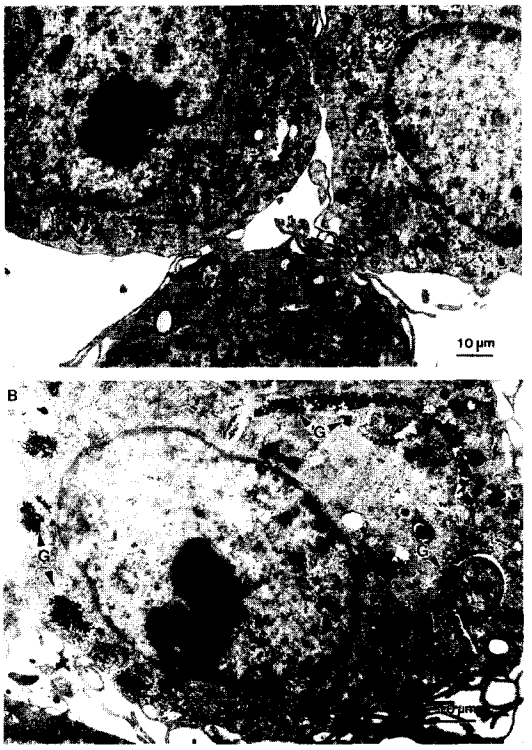

Fig. 2. The ultrastructure of J774 macrophages from (A) a control culture and (B) J774 macrophages treated with JM 2820 at a concentration of 50 μg/ml for 48 h. G illustrates electron dense granules. In contrast to the control cell, large amounts of high electron dense granules were observed in the vacuoles and ramifying system of tubular cavities.

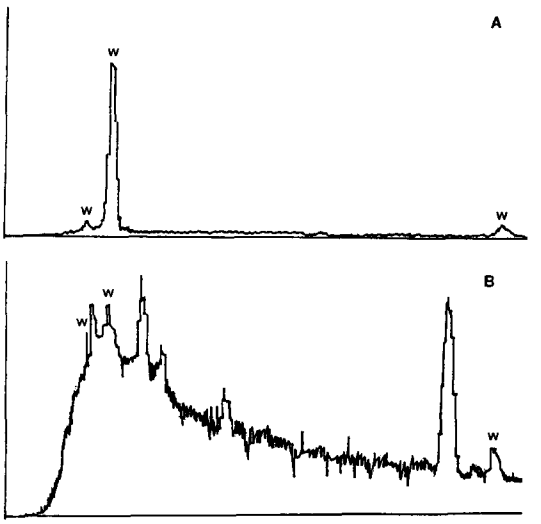

Fig. 3. (A) SEM microprobe with WDS standard tungsten X-ray spectrum. The vertical full scale (VFS) of this spectrum is 512 X-ray counts and the spectrum was collected over 20 s. (B) SEM microprobe with WDS X-ray spectrum obtained from J774 cells incubated with JM 2820. The VFS of this spectrum is 128 X-ray counts and the spectrum was collected over 600 s. A series of spectral peaks characteristic of tungsten (W) are indicated. With the detection limition of 0.179, 0.45% of atom was identified as tungsten.

fell below the detection limitation (10^{-16} g of an element) of TEM–EDS (Bozzola and Russell, 1992). Indeed, only 18 tungsten atoms exist in each JM 2820 molecule. However, it is clear from the electron microprobe WDS X-ray microanalysis that JM 2820 did associate with the macrophages, even though the amount of tungsten in the cells was very small. The ability to detect tungsten in JM 2820-treated cells is most likely enhanced by the electron microprobe WDS and superposition of several tungsten deposits aligned in the cytoplasm. Although WDS X-ray microanalysis is not often used in biological samples, it has some advantages over EDS (Bozzola and Russell, 1992). WDS offers an approximately 10-fold better capability than EDS to discriminate between closely spaced X-ray energy peaks. It is also more suitable for detection of trace amounts of elements. Electron microprobe WDS was not able to detect the tungsten in J774 macrophages treated with JM 1591 and JM 1596, probably

because the amount of tungsten associated with the cells was below the limit of detection. These results are consistent with those of an in vivo study which demonstrated that following intravenous administration, the greatest polyoxometalate accumulation in tissues was observed with JM 2820 (Ni et al., 1994). Further, Berry and Galle (1990) reported that HPA-23, a heteropolytungstate, was concentrated in the lysosomes and localized in the macrophages of different tissues, including kidney, thymus, spleen and bone marrow.

The results of the present study demonstrate that polyoxometalates accumulate in macrophages. In AIDS, human immunodeficiency virus proliferates in the macrophages before being released to infect other cells (Maddon et al., 1986; Gartner et al., 1986). Thus, the anti-HIV activity of polyoxometalates may, in part, be associated with their cellular localization.

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