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Biological activity and redistribution of nucleolar proteins of two different cell lines treated with *cis*-dichloro-1,2-propylenediamine-*N,N,N′,N′*-tetraacetato ruthenium (III) (RAP)

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The interaction of a newly synthesized antitumor complex *cis*-dichloro-1,2-propylenediamine-*N,N,N′,N′*-tetraacetato ruthenium (III) (RAP) with DNA was investigated *in vitro* through a number of techniques including comet assay, immunoprecipitation, and immunolocalization of certain nucleolar proteins (the upstream binding factor (UBF) and fibrillarin) involved in DNA transcription, rRNA processing, and ribosomal assembly. The results showed that RAP binds to the DNA of two cell lines (H4 and Hs-683) causing a delay in cell proliferation rate leading to a number of cellular modifications. These modifications include DNA-damage assessed by the single cell gel electrophoresis method (comet assay) and variation in the expression of nucleolar proteins; UBF was more abundant in RAP treated cells, this was explained by the high affinity of this protein to DNA modified by RAP. On the other hand, fibrillarin was found in less quantities in RAP treated cells which was explained by a de-regulation of the ribosomal machinery caused by RAP.

Keywords: Antitumor drugs, DNA-damage, Fibrillarin, RAP, Tumor cells *in vitro*, UBF

Complexes based on ruthenium, one of the platinum group metals, have been more studied in recent years because of their potential antitumor activity[1-3]. Biochemical studies demonstrated that Ru (III) complexes bind tightly to proteins[4]. A new generation of Ru (III) complexes with polyaminocarboxylic molecules as chelating agents were investigated in order to find water-soluble ruthenium compounds with antitumor activity; the resulting complex: $[RuLCl_2]H.4H_2O$ (RAP) (Fig. 1), where L is 1,2-propylediammine-*N,N,N′,N′*-tetraacetic acid (PDTA), consisting of an octahedral molecule with two chlorine atoms in a *cis* conformation and two PDTA ligands; this complex was characterized by a very low toxicity and a high solubility in water[5]. It was shown that RAP induced a distortion of the DNA helix explained by an interaction between RAP and DNA resulting in the formation of DNA adducts[6]. RAP induced a low cytotoxicity in TG cultured tumor cells, as well as, DNA-damage showed by the formation of comets when RAP-treated TG cells were subjected to electrophoresis under alkaline conditions. Studies on metaphase plates did not show any clastogenic effect produced by RAP-adducts on cultured tumor cells *in vitro*[6].

The nucleolus constitutes a highly dynamic substructure of the nucleus and is the site of rDNA transcription, rRNA maturation and ribosome production[7]; it's also the site of many other RNA processing reactions and viral maturation and function[8-11]. Nucleolar alterations occur in many pathologies and are, for example, of diagnostic importance in cancer[12]. Electron microscopy studies revealed that the nucleolus consists of three different regions: fibrillar centers (FCs) lighter areas surrounded by a highly contrasted region, the dense fibrillar component (DFC), and the granular component (GC) in which the FCs and DFC are embedded[13]. Ribosomal RNA transcription apparently takes place within the DFC[14], possibly at the border with the FCs[15,16]. The nucleolus contains a large

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variety of proteins, including the upstream binding factor (UBF) and fibrillarin.

UBF was found to be linked to the regulation of the rRNA gene locus; this factor belongs to the sequence-non specific class of high mobility group (HMG) proteins that appear to function exclusively in polymerase I transcription[17]. By recognising the rRNA gene promoter together with the transcription factor SLI, UBF facilitates the transcription of 5.8S, 18S, and 28S rRNAs[18]. It is also known as the nucleolar organizer region (NOR) autoantigen NOR-90[19] detected by antisera from patients with autoimmune diseases[20]. UBF was found to be highly conserved among various vertebrates[21,22]. NOR-90/UBF has two isoforms, NOR-97 (UBF_1) and NOR-94 (UBF_2); NOR-94 differs from NOR-97 by lacking 37 aminoacids within the second High Mobility Group (HMG) box which reduces the DNA-binding capacity of this domain[23].

Fibrillarin is a 34 kDa protein that derives its name from its localization to both the FCs and the DFC of the nucleolus; it was first identified by human autoimmune sera from patients with scleroderma[24,25]. Fibrillarin is associated with U3 and other small nucleolar RNAs (snoRNAs) required for rRNA processing[19]. This protein is directly involved in many post transcriptional processes including pre-rRNA processing, pre-rRNA methylation, and ribosome assembly[20]. Fibrillarin is highly conserved in sequence, structure, and function in eukaryotes[21,22,26], and was originally identified as the enzymatic component of the C/D class of snoRNPs which is responsible for site-specific 2'-*O*-methylation of ribose[27].

The aim of the present work is to study the modifications involved in two human cancer cell lines, H4 and Hs-683, following a continuous treatment with RAP; investigate the drug cytotoxicity, the damage it can cause to the DNA of these cell lines, and the expression pattern of some proteins involved in transcription and RNA processing machinery of the cell.

Materials and Methods

Chemicals—RAP compound (Fig. 1) was dissolved in water to the desired concentration and stored at 4 °C.

Human Sera—NOR-90/UBF antibody was obtained from patients with rheumatoid arthritis. Anti-fibrillarin antibody was obtained from patients with Scleroderma.

Cell Culture—Human neuroglioma H4 cells and glioblastoma Hs-683 cells were grown and maintained in cell culture flasks containing Dulbecco's modified Eagle medium (DMEN) supplemented with 10% foetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 100.0 U/mL penicillin-streptomycin, 1% fungizone, and 1% anti-PPLO. Cells were treated with RAP at 1, 10, and 100, and 1000 µg/mL for 6 days. Culture medium for both control and treated cells was changed every day. Cells were trypsinized from culture flasks and counted in a Thomas chamber.

For all the following experiments, cells were treated with RAP at 100 µg/mL for 4 days by changing the culture medium every day. For radioactive labelling, ^{35}[S] methionine-cystein (*Trans* ^{35}S-label) (1000-1100 Ci7 mmol, ICN Radiochemicals, Cleveland, USA) was added at the concentration of 50 µCi/mL on the 3^{d} day of RAP treatment.

Before each experiment, cells were trypsinized from culture flasks, washed with PBS (136 mM NaCl, 2.68 mM KCl, 6.7mM Na_2HPO_4, and 1.46 mM KH_2PO_4) and pelleted after a mild centrifugation. Cells were then counted in a Thomas chamber to have the same number of starting material in both control and RAP treated cells. Data were analyzed statistically using Student's *t*-test. A difference of $P< 0.001$ was considered statistically significant.

Single cell gel electrophoresis (Comet assay)—DNA breaks were detected using an adaptation of the method of Singh *et al*.[28]. Alkaline treatment followed by electrophoresis at high *p*H results in streaming of cellular DNA toward the anode, giving the appearance of the tail of a comet, this effect is seen only when DNA contains breaks or lesions converted to breaks under alkaline conditions. The diamidino-2-phenylindole (DAPI) (Sigma) staining

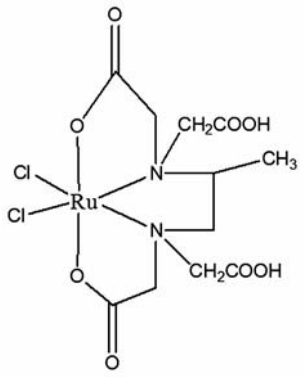

Fig. 1—Structure of RAP

intensity was taken as a measure of the extend of DNA breakage[29].

After 4 days of treatment with RAP at 100 µg/mL, H4 and Hs-683 cells were suspended in low melting point agarose in PBS, *p*H 7.4 at 37 °C and pipetted onto frosted microscope slides pre-coated with a layer of normal melting point agarose. Slides were chilled on ice for 10 min then immersed in lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, *p*H 10.0, 0.2 mM NaOH, 10% DMSO, and 1% Triton) at 4 °C for 1h to remove cellular proteins. Slides were then placed in a 270 mm wide electrophoresis tank containing 300 mM NaOH and 1 mM Na_2EDTA, *p*H 10.0 for 30 min. Electrophoresis was carried out at 27V for 20 min at 12 °C. The slides were then washed 3 times, 5 min each, with 0.4 M Tris-HCl, *p*H 7.5 at room temperature. Once dried, the slides were fixed in absolute methanol for 15 sec and stained with 5 µg/mL DAPI. Slides were then examined under fluorescent microscope; 20 cells from each treatment were digitalized and analyzed using an image analysis software (PCBas Software). The images obtained were used to estimate the DNA content in the individual nuclei and to evaluate the degree of DNA damage representing the fraction of total DNA in the tail. Cells were classified into five classes: 0 (< 7% of the DNA in the tail, undamaged), 1 (8-15%), 2 (16-22%), 3 (23-30%), and 4 (> 30%, maximally damaged cells).

Immunoprecipitation—H4 and Hs-683 Cells were treated for 4 days with RAP at 100 µg/ml, cells were incubated on the 3^d day with methionine-cystein labelled with ^{35}[S]. Immunoprecipitation was carried out using protein A-bound Sepharose CL-4B (Sigma). Extracts from ^{35}[S] methionine-cystein labelled H4 and Hs-683 cells were prepared using NET buffer (50 mM Tris-HCl, *p*H 8, 400 mM NaCl, 5 mM Na_2EDTA, 1% Triton X-100, and 0.05% azide) containing protease inhibitors (soybean trypsin inhibitor, aprotinin, benzamidine, CLAP mixture, and 100 mM PMSF in methanol) and sonicated for 1-2 min to allow the breakage of cell membranes. Extracts were then incubated with the anti-NOR90/UBF or the anti-fibrillarin antibodies coupled to Sepharose for 90 min. The immunoprecipitates were washed 3 times in NET buffer, 3 times in NET buffer containing 800 mM NaCl and twice in a solution containing 10 mM Tris-HCl, *p*H 8, and 1 mM Na_2EDTA. The antibodies were eluted by adding dithiotreitol (Sigma) and SDS-PAGE sample buffer after a 10 min warming at 98 °C. Electrophoresis was performed as described by Laemmli[30] on a 12% SDS-polyacrilamide gel using the mini Protean II electrophoresis cell (Bio-Rad, Richmond, CA).

Gels were treated with a fixing solution containing isopropanol-distilled water-acetic acid (25:65:10) for 1h with low agitation. For the detection of radiolabelled proteins, gels were soaked in Amplify (Amersham International, Buckinghamshire, England) with agitation for 30 min. Gels were dried under vacuum at 80 °C using a model 583 gel dryer and then exposed in an imaging plate (20-40) for 2 h; the exposure time depends on the intensity of the radiation. The plate was read in an IP-Autoradiography System Bio-Imaging Analyser (Fujix, Bas1000). Quantification of the bands was performed using PCBas Software.

Immunocytochemical labelling for electron microscopy—H4 and Hs-683 cells grown and maintained in Petri dishes for 4 days with continuous treatment with RAP at 100 µg/mL, were processed for the immunocytochemical electron microscopic studies. Cells were fixed in a mixture of 4% paraformaldehyde and 1.6% glutaraldehyde in 0.1 M cacodylate buffer, *p*H 7.3 for one hour at 4 °C; all fixations were performed in Petri dishes. Subsequently, cells were scrubbed with a rubber policeman and pelleted by centrifugation. Samples were washed in 0.1 M cacodylate buffer, *p*H 7.3 and incubated in 0.1% uranyl acetate in acetone for 4 h at 4 °C to block free aldehyde groups; cells were then washed in the same buffer again. Low temperature embedding with the hydrophilic resin Lowicryl K4M (Chemische Werke Lowi., Waldkraiburg, FRG) was performed with slight modifications according to the method described by Roth[31]. Samples were dehydrated in a series of graded methanol at progressively lower temperatures; during infiltration with Lowicryl K4M at -20 °C, 90% methanol was used as the dehydrating agent. Lowicryl was polymerized by indirect long wave (360 nm) UV irradiation from a 15 Watt Phillips fluorescent lamp for 24 h. Ultrathin sections were cut on a Reichert-Jung Ultracut E ultramicrotome and mounted on 300-mesh nickel grids.

Grids were incubated by floating them, cell section down, on a drop of PBTT (0.1 M PBS, *p*H 7.4, 1% BSA, 0.05% Triton X-100, and 0.05% Tween 20) for

30 min at room temperature and then washed in PBS. Grids were incubated with anti-NOR90/UBF or anti-fibrillarin antibodies diluted in PBS for 1 h at room temperature. After a wash in PBS, the grids were incubated in protein A-gold complex prepared following the method of Bendayan[32] (the protein A-gold complex was diluted in PBTT until an absorbency of 0.06 at 525 nm was reached) for 1 h at room temperature. The grids were washed with PBS followed by another wash with distilled water and then contrasted with 2% uranyl acetate in distilled water. To demonstrate the specificity of the labelling, controls were performed by omitting the incubation with the primary antibodies.

Results

Interaction of RAP with cultured tumor cells—H4 and Hs-683 cells were subjected to 6 days continuous treatment with RAP at 1, 10, 100, and 1000 µg/mL by changing the culture medium each day. Figure 2 shows the evolution with time of the total number of cultured H4 and Hs-683 cells in the absence (control) and presence of RAP for both H4 and Hs-683 cell lines. At 1 and 10 µg/mL concentration of RAP, both cell lines did not show any statistical significance in comparison with the controls (Fig. 2). H4 cells have a cell cycle of approximately 28 h which was increased to 29 h after treating cells with RAP at 100 µg/mL; a significant delay in the proliferation rate was observed after 96 h of continuous treatment with RAP at 100 µg/mL (Fig. 2a). Hs-683 cells have a cell cycle of approximately 33-34 h increased to 35 h after treatment with RAP at 100 µg/mL; a significant delay in the proliferation rate was observed after 144 h of continuous RAP treatment at 100 µg/mL (Fig. 2b), H4 cells seem to be more sensitive to RAP than Hs-683. Neither of the cell lines show any changes in their morphology after RAP treatment. When cells from both cell lines were treated with RAP at 1000 µg/mL, figures of necrosis were shown and the cells started to die after 24 h of treatment (Fig. 2a and b). Data were analyzed statistically using the Student's *t*-test. A difference of $P< 0.001$ was considered statistically significant.

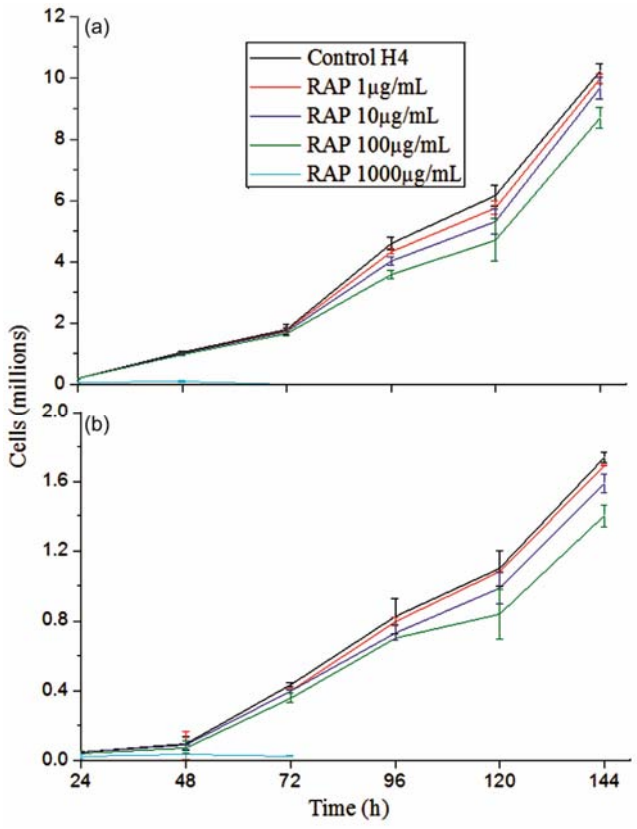

Fig. 2—Effect exerted by RAP on the kinetic of proliferating H4 (a) and Hs-683 (b) cells.

DNA-damage caused by RAP on human cultured cells—To assess the DNA damage caused by RAP, the comet assay was applied, a single cell gel electrophoresis technique able to detect migration of DNA from individual cell nuclei following alkaline treatment[28]. The DNA damage detected by the alkaline assay can rise through multiple mechanisms, including DNA:DNA cross-linking, DNA single strand breaks, alkali labile sites, and incomplete excision repair sites present at the time of lysis[28]. For each cell, total fluorescence, as well as, nuclear fluorescence were scored in order to obtain the percentage of tail staining, being this parameter directly related to damaged DNA. The fraction of DNA in the tail has been reported to be a good method for evaluating DNA damage[29]. Figure 3

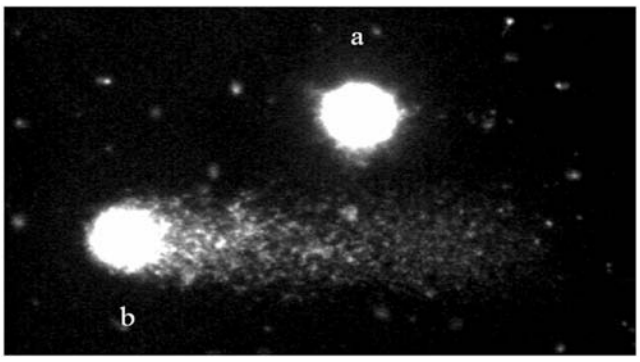

Fig. 3—Human H4 cells. Cells were isolated and subjected to single-cell gel assay. Following electrophoresis, cells were stained with DAPI: (a) control H4 cells and (b) treated H4 cells.

shows the picture of two H4 cells, control and treated with RAP at 100 µg/mL, the treated cell shows a figure of a comet with its tail representing the fraction of damaged DNA that migrated when subjected to electrophoresis. Cells were distributed according to the percentage of damaged DNA in five classes ranging from 0 (undamaged cells) to 4 (maximally damaged cells).

Figure 4 shows the distribution of cells according to their damaged DNA, in Hs-683 control most cells were undamaged (73.68%), after 4 days of RAP continuous treatment at 100 µg/mL most cells were significantly damaged with 50% cells with maximal damage, and 38.14% cells with damage assessed between 15-30% (Fig. 4a). In H4 cells, in the control, most cells were undamaged (82.5%); after treatments with RAP at 100 µg/mL for 4 days, most cells were maximally damaged (71.25%), and 21.25% cells with damage assessed between 15-30% (Fig. 4b). Those results show that H4 cell line seems to be more sensitive to RAP than Hs-683 cell line in causing more damage to the DNA of the cells.

Effect of RAP on nucleolar proteins—An immunoprecipitation assay of two nucleolar proteins was carried out: the first protein the NOR-90/hUBF corresponding to the nucleolar transcription factor hUBF (human upstream binding factor), this protein exists in two molecular forms: UBF_1 and UBF_2 reported to be 97 and 94 kDa respectively. The second protein is fibrillarin having a molecular weight of 34 kDa. We preceded to immunoprecipitate the anti-NOR-90/hUBF and anti-fibrillarin antibodies after radiolabelling them with *trans* [^{35}S] methionine-cystein. A mono-dimensional gel electrophoresis of the whole radioactive cell extracts obtained from control and RAP treated cells during 4 days was carried out. After analyzing the images, it was found that the signal corresponding to UBF in H4 treated cell extracts was higher than the controls (Fig. 5a), also a higher increase in UBF_1 expression was registered compared to UBF_2 in RAP treated cell extracts. The optical density of the bands corresponding to UBF_1 and UBF_2 in control (86308.76±6145.11 and 76252.55±418.12) and RAP treated (111620.8±5386.87 and 93797.82±317.94) cells showed an increase in the protein expression with 1.29-fold higher for UBF_1 and 1.23-fold higher for UBF_2 in RAP treated cells compared with the controls. For Hs-683 cells, the same pattern was observed for UBF expression as for H4 cells with an increase in the density of the band corresponding to UBF in cells treated with RAP higher than the

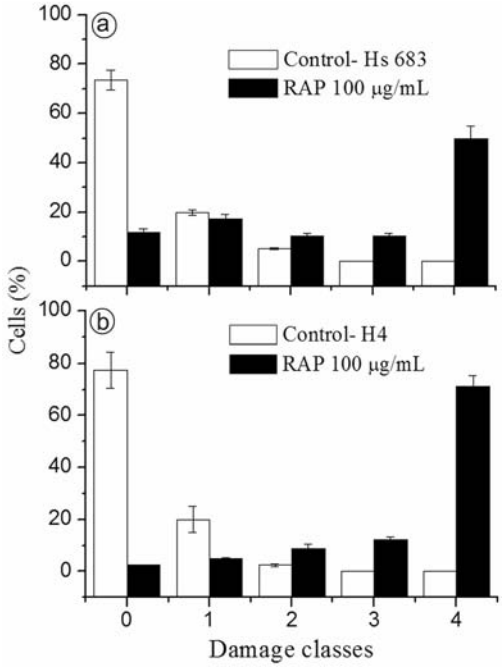

Fig. 4—Comet assay. Damage observed after 4 days of continuous treatment with RAP at 100 µg/mL in Hs-683 cells: control and RAP treated cells (a); and in H4 cells: control and RAP treated cells (b).

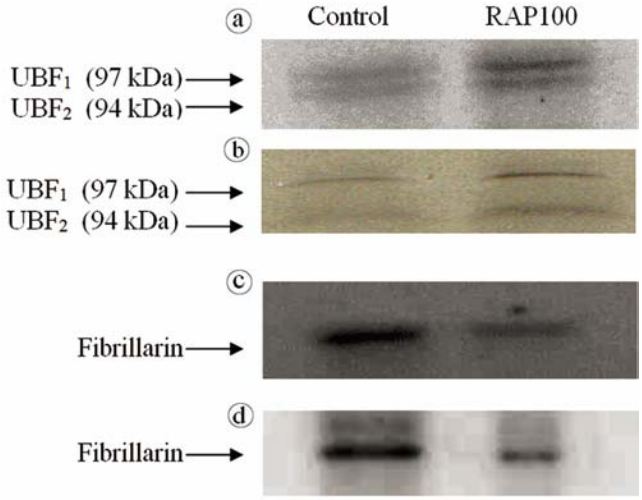

Fig. 5—Immunoprecipitation of the human transcription factor hUBF an fibrillarin. H4 and Hs-683 cells were treated with 100 µg/mL RAP for 4 days; cells were incubated on the 3th day with methionine-cystein labelled with ^{35}[S]; cellular lysates were immunoprecipitated and run on an SDS-PAGE electrophoresis gel. Gels were dried and exposed in an imaging plate (20-40) for 2 h. Quantification of the bands was performed using a PCBas Software computer. (a) H4 incubated with anti-NOR/UBF, (b) Hs-683 incubated with anti-NOR/UBF, (c) H4 incubated with anti-fibrillarin and, (d) Hs-683 incubated with anti-fibrillarin.

control, also an increase in the UBF_1 isoform was detected in comparison with the UBF_2 isoform (Fig. 5b). The optical density of the bands corresponding to UBF_1 and UBF_2 in control (75135.16±4019.421 and 58869.68±4720.305) and RAP treated (121217.5±7156.352 and 83649.22±2697.91) cells showed 1.61-fold increase in UBF_1 and 1.42-fold increase in UBF_2 respectively in RAP treated cells compared with the untreated cells.

For the fibrillarin, we found that the band corresponding to that protein in control H4 cell extracts was more dense that the one corresponding to the RAP treated ones (Fig. 5c). The optical density of the band corresponding to Fibrillarin in both control and RAP treated cells showed 0.38-fold decrease in the protein expression in RAP treated (20967.74±1725.935) cells compared with the controls (54592.19±7538.982). In Hs-683, the same pattern of band intensity was observed as shown for H4 with high dense band in control extracts compared to the treated ones (Fig. 5d). The optical density of the bands corresponding to Fibrillarin in both control and RAP treated cells with 0.39-fold decrease in the band intensity in the treated cells (26276.4±6010.252) compared with the controls (65841.12±8178.793).

The immunodetection was quantified with anti-NOR and anti-fibrillarin sera in different nucleolar sub-compartments of the control and RAP treated H4 and Hs-683 cells simultaneously.

For H4 cells, the obtained results in the control demonstrated, as shown in other cell lines, that the UBF factor immunolocalizes with anti-NOR serum preferentially in the fibrillar centres and in the dense fibrillar component; in the granular component and in the nucleoplasm its localization was lower. In RAP treated cells, an immunolocalization of this factor similar to the one found in the controls was observed, but with a subsequent increase in its amount in the fibrillar centres which was 1.31 times higher than in the controls (Fig. 6a). These results were coherent to those obtained after immunoprecipitation.

The immunodetection of fibrillarin was quantified within the different nucleolar sub-compartments of the control and treated H4 cells. Although this protein is fundamentally present in the DFC, it was also found in the other sub-compartments of the nucleus[33]. The obtained results in the control showed, as found in other cell lines, that fibrillarin immunolocalizes in the dense fibrillar component, in the granular component and in the nucleoplasm; it was also detected in the FCs but in low quantities. In RAP treated cells, the same pattern of distribution of this protein around the above mentioned components was observed with a significant decrease in its presence in the nucleoplasm, DFC, and in the GC with 0.5-fold, 0.66-fold, and 0.7-fold decrease in its content respectively (Fig. 6c). These results were coherent with those obtained after immunoprecipitation.

For Hs-683 treated cells, as shown in H4 treated cells, UBF was found to be significantly over-expressed in the FCs with 1.55-fold increase as compared with the controls (Fig. 6b), in the other nuclear sub-compartments, there were the same distribution of the protein in both treated and untreated cells.

The immunolocalization of Fibrillarin in Hs-683 cells was similar to that shown in H4 cell line with lower quantities of that protein within the nucleoplasm, GCs, and the DFC with amounts decreasing 0.5, 0.46, and 0.46 times in the treated cells compared with the controls (Fig. 6d).

Discussion

The cellular expression of RAP-DNA interaction was demonstrated earlier using an ovarian carcinoma cell line (TG), where it was shown that RAP induced a significant decrease in the proliferation rate after 48 h of continuous drug treatment[6]. Using H4 cells, RAP at a concentration of 100 µg/mL was found to cause again a significant decrease in cell number count but after 96 h of drug treatment; Hs-683 cell line showed the same pattern of growth kinetics as H4 cells with a significant delay in proliferation rate after 144 h of RAP treatment at a concentration of 100 µg/mL. TG cells seemed to be more sensitive to RAP than H4 and Hs-683 cells. This delay in the growth kinetic was explained by DNA-damage caused by RAP when incorporated into the cells; the comet assay was performed in order to confirm these findings and to assess the DNA-damage caused by RAP on cultured cells *in vitro*, the assay is based on the detection of DNA migration from individual cell nuclei after alkaline treatment. In both cell lines, the controls showed around 80% of undamaged cells, whereas after RAP treatment, H4 showed 70% of cells and Hs-683 showed 50% of cells that were maximally damaged; these results confirm the fact that RAP effectively causes DNA-damage. Studies using TG cell line treated with the same compound showed the same results were most cells were maximally damaged[6]. H4 cells seem to be more

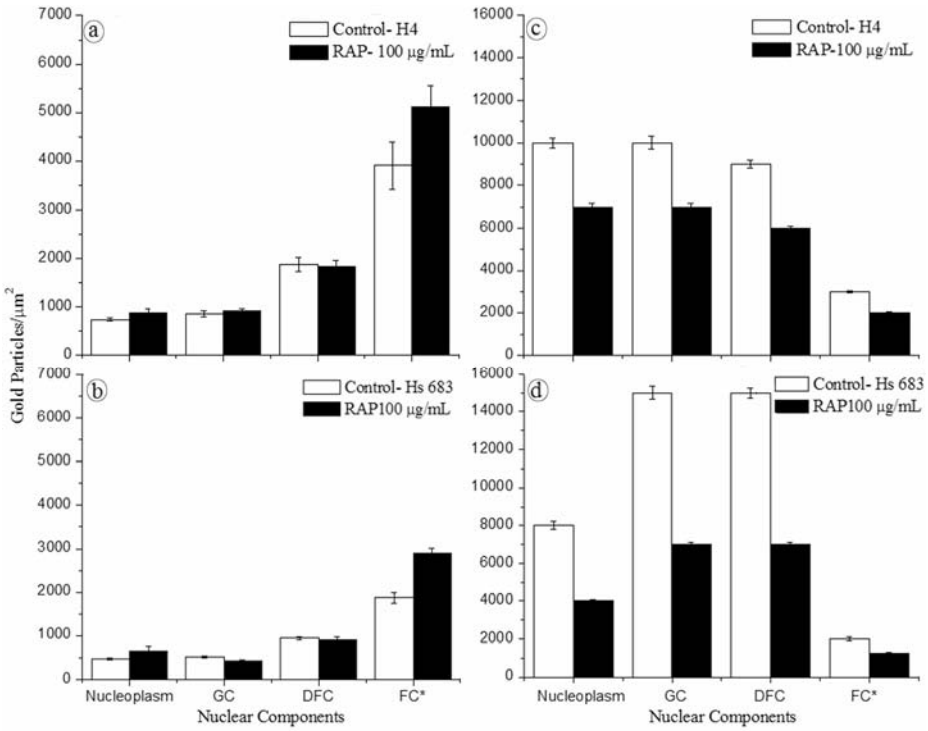

Fig. 6—Quantification of the immunocytochemical staining to detect the NOR-90/UBF and fibrillatin respectively. H4 and Hs-683 cells were treated for 4 days with RAP at 100 µg/mL, cells were embedded in Lowicryl resin, cut in ultrathin sections and mounted onto nickel grids. The grids were then incubated with anti-NOR serum or anti-fibrillarin serum and the gold particles were counted under electron microscopy. The quantification was expressed as the density of gold particles/µm^2 within the different nucleolar compartments. GC–Granular component, DFC–Dense fibrillar component and FC–Fibrillar centres. (a) H4 cells incubated with anti-NOR serum; (b) Hs-683 cells incubated with anti-NOR serum; (c) H4 cells incubated with anti-fibrillarin serum; (d) Hs-683 cells incubated with anti-fibrillarin serum. *Significant difference at $P< 0.01$.

sensitive to RAP than Hs-693 cells by showing more damaged cells using the comet assay, as well as, by showing more significant delay in cell proliferation than Hs-683 cells. Previous studies on RAP-DNA interaction using the bromodeoxyuridine (BrdU) assay which consists in the incorporation of the pyrimidine analogue BrdU instead of tymidine into DNA of proliferating TG cells *in vitro*, the results showed a significantly high incorporation of BrdU in RAP treated cells compared to the untreated cells explained as an expression of repair mechanisms activated by DNA damage[6].

The nucleolus is a highly structured and dynamic organelle involved in the transcription and maturation of rRNA and ribosome biogenesis as well as in apoptosis and in cell cycle control[34]. The present evidence suggests that the fibrillar center (FC) represents pools of factors required for transcription and pre-rRNA processing together with non transcribed rDNA[8]. rRNA synthesis requires RNA pol I and other factors[35]. In mammals, these are UBF and selectivity factor SLI. More recent studies found that UBF binds throughout the transcribed region and forms a distinct rRNA gene chromatin through which RNA pol I must transcribe[36]. Fibrillarin was found to be important to the RNA formation and processing machinery; it is a highly conserved nucleolar protein that is associated with box C/D snoRNAs and functions in early processing and modification of pre-rRNA[21,37], ribosome assembly[20], and also is essential for early embryonic development[38]. Electron microscopy and immunocytochemical studies showed that fibrillarin plays a role in nucleolar assembly by packaging pre-nuclear bodies[39], also, fibrillarin was found to be critically involved in the maintenance of nuclear shape and cellular growth[40].

In this paper, the possible alterations induced by RAP at the protein level was studied. The results obtained after immunoprecipitating UBF in the two cell lines studied showed that UBF expression followed the same pattern in both cell lines by being highly expressed in RAP treated cells in comparison with the controls. The same results were found using TG cell line treated with RAP[41]; in

another study, UBF was found to be more expressed in cells treated by cisplatin[42,43]. Also, it was found that UBF_1 expression was higher in both cell lines than UBF_2 this could be explained by the fact that UBF_2 was five fold less active according to a study done by Hannan *et al*.[44]; Stefanovsky and Moss[23] demonstrated that UBF_1 and UBF_2 possess distinct abilities to regulate the transcription elongation rate of RNA pol I by finding that UBF_2 was less efficient than UBF_1 at arresting the RNA pol I transcription complex. Another study in which small interfering RNA (siRNA) knock-down of UBF caused a major reduction in the number of active rRNA genes and induced cell cycle arrest and that UBF_1 but not UBF_2 was required to maintain the rRNA genes active[45].

Fibrillarin was found in lower quantities in H4 and Hs-683 treated cells in comparison with the controls. These results were in accordance with those found by Delmani *et al*.[41] using the same antiumor drug (RAP) on TG cells; they found that UBF was highly expressed and fibrillarin was found in fewer quantities in treated cells; also, they showed by quantifying the whole protein extracts from TG cells that RAP treated extracts were expressing more high molecular weight proteins and less medium to low molecular weight proteins in comparison with the untreated cells[41]. Since UBF is expressed in higher quantities in cells treated by cisplatin or RAP, this was explained by the fact that adducts formed, as a result of cross-links between the drug and DNA, interact with proteins that contain HMG domains such as UBF; and because UBF is an essential transcription factor for RNA pol I, it was suggested that rRNA synthesis may be disrupted if the adducts hijack hUBF[43].

An immunocychemical study was carried out under electron microscopy about the relative content of UBF and fibrillarin in control and RAP treated cells. The results showed a major abundance of UBF in RAP treated cells compared with the untreated cells. In this work, as shown in other cell lines, it was found in the controls that UBF immunolocalizes preferentially in FCs and in the DFC, while, it was found in fewer quantities in the GC and nucleoplasm. In RAP treated cells, there was a similar redistribution of UBF immunolocalization as shown in the controls with a significant increase in its presence in the FCs in RAP treated cells for both cell lines; although there was a slight increase in UBF content in the nucleoplasm of both cell lines. The same results were found using TG cells treated with RAP with a significant increase in UBF content in the nucleoplasm[41], other researchers observed a redistribution of UBF within the nucleus from being predominantly nucleolar to a dispersed nucleolar/nuclear/cytoplasmic presence after serum starvation[46]. Cisplatin also causes a redistribution of UBF as well as other components needed for RNA pol I transcription by blocking rRNA synthesis[43].

Fibrillarin is a 34 kDa protein that derives its name from its localization to both the FCs and DFC of the nucleolus[24]. Other studies localized fibrillarin in the GC and DFC but little in the FCs[47]. The present studies localized fibrillarin within the DFC, GC, and nucleoplasm with significantly high quantities in the controls compared with RAP treated samples in both cell lines, the FCs also showed the presence of fibrillarin but with no significant quantities in control and RAP treated cells in both cell lines. These results are consistant with those found using TG cells treated with RAP[41]. Also, it was found that injecting anti-RNA polymerase I antibodies into the nuclei lead to the inhibition of nucleolar transcription[48]. Treating cells with subtoxic concentrations of $HgCl_2$ caused a redistribution of fibrillarin from the nucleolus to nucleoplasmic aggregates[49]; also, a treatment with mercury causes a redistribution of fibrillarin leading to its co-localization with nucleoplasmic proteasomes and proteasome-dependent processing of fibrillarin within nuclei[50]. The present results are, indeed, in good agreement with those findings suggesting that RAP induces an alteration of the protein behaviour in cultured cells *in vitro*.

It was previously found that RAP causes a distortion of the helical chain of DNA leading to the formation of DNA-adducts[6], it was also found that RAP causes a modification of the protein profile[41]. In this communication, the findings using other cell lines which suggest that RAP acts in the same manner in different cell lines *in vitro* are confirmed. In conclusion, the present data suggests that RAP-DNA adducts cause a modification in the protein profile pattern in different cell lines by decreasing the expression of low molecular weight proteins and increasing the expression of high molecular weight proteins. The study of the UBF was based on its role in activating transcription and the up-regulation of this protein, as a result of RAP-DNA interaction, could be an indicative of an eventual repair process needing more UBF to initiate the transcription machinery of the cell. Whereas for fibrillarin, the low

presence of this protein could be the result of a growth arrest induced by RAP causing a down-regulation of ribosome synthesis that decreased the need for pre-rRNA processing factors in the nucleolus.

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