Original Article

The Effect of a Histone Deacetylase Inhibitor – Valproic Acid – on Nucleoli in Human Leukaemic Myeloblasts

(nucleoli / histone deacetylase inhibitor / leukaemic myeloblasts)

K. SMETANA[1], M. ZÁPOTOCKÝ[2]

[1]Institute of Haematology and Blood Transfusion, Prague, Czech Republic
[2]Charles University in Prague, 2nd Faculty of Medicine, Prague, Czech Republic

Abstract. The present study was undertaken to provide more information on nucleolar changes induced by a histone deacetylase inhibitor such as valproic acid in leukaemic myeloblasts at the single-cell level. For this study, RNA in nucleoli was visualized by a simple but sensitive cytochemical procedure in unfixed cytospins of short-term bone marrow cultures from patients suffering from acute myeloid leukaemia. Valproic acid in leukaemic myeloblasts markedly reduced the nucleolar size and also produced significant transformation of "active" to "resting" and "inactive" nucleoli that reflected the alteration of the nucleolar transcription in sensitive myeloblasts. On this occasion it should be added that valproic acid significantly increased the incidence of altered myeloblasts that changed to apoptotic cells or apoptotic bodies and cell ghosts. In contrast to the above-mentioned decreased nucleolar size, the nucleolar RNA concentration, expressed by computer-assisted RNA image densitometry in valproic acid-treated myeloblasts, was not significantly changed. The results of the present study clearly indicated that the nucleolar size and transformation of "active" to "sleeping" or "inactive" nucleoli are convenient markers of the sensitivity and alteration of leukaemic myeloblasts produced by a histone deacetylase inhibitor, valproic acid, at the single-cell level.

Introduction

Previous studies clearly demonstrated that histone deacetylase inhibitors (HDACIs), including valproic acid or sodium valproate (VPA), alter gene expression and act as cytostatic agents that inhibit cell growth, influence cell differentiation and induce the apoptotic process. On the other hand, the mechanisms of HDACI action are less understood although they apparently act on nuclear chromatin structure and gene transcription (Blaheta et al., 2005; Han et al., 2006; Santini et al., 2007; Smetana et al., 2007, 2008a; Emanuele et al., 2008; Eot-Houllier, 2009; Hrebackova et al., 2010; Papi et al., 2010; Tan et al., 2010). The anti-tumour effects of VPA were noted on a variety of malignant tumours including established cell cultures of malignant myeloblasts (Han et al., 2006; Santini et al., 2007; Smetana et al., 2007; Duenas-Gonzalez et al., 2008; Hrebackova et al., 2010; Papi et al., 2010; Tan et al., 2010). However, the direct effects of VPA on the cell nucleolus of malignant cells were less studied (Smetana et al., 2007), although these nuclear components are very convenient markers of cell states at the single-cell level (Smetana, 2002, 2005).

The present study was undertaken to provide more information on the effect of VPA on nucleolar bodies of malignant cells and short-term cultures of myeloblasts from selected patients suffering from acute myeloid leukaemia. They appeared to be a very convenient model for such studies because they possessed a satisfactory number of these cells for such study. On this occasion it should be briefly mentioned that nucleoli are gene-rich multifunctional cell organelles that participate in cell proliferation, differentiation, maturation, aging and cell death (Busch and Smetana, 1970; Pederson, 1998; Olson et al, 2000; Smetana, 2005; Boisvert et al., 2007). They are also sites of the ribosomal RNA transcription and assembly of ribosomal particles that migrate to the cytoplasm and represent a substantial part of the cell proteosynthetic machinery (Busch and Smetana, 1970; Fakan and Puvion, 1980; Wachtler and Stahl, 1993; Hozák, 1995; Tschochner and Hurt, 2003; Raška et al., 2006).

The results of the present study clearly demonstrated that a HDACI – VPA – significantly altered the nucleolar structural organization. The decreased nucleolar size and decreased incidence of myeloblasts with "active" nucleoli reflected alteration of the main nucleolar functional activities. In addition, the reduced nucleolar size without a decrease of the nucleolar RNA concentration apparently indicates that the nucleolar RNA content in VPA-treated myeloblasts depends on the nucleolar size.

Material and Methods

Nucleoli, i.e. nucleolar bodies without perinucleolar chromatin, were studied in cytospins of short-term bone marrow cultures of 7, 13 and 17 years old female patients suffering from acute myeloid leukaemia without and with addition of a HDACI – VPA – for 24 and 48 h. These patients were selected for the present study because their bone marrow contained a very large percentage of myeloblasts (57–75 %), some of which possessed Auer bodies. The bone marrow blood samples of these untreated patients with cytostatic therapy were taken for diagnostic purposes and the ethics committee of the Institute approved the protocols for the present study. The bone marrow samples were cultured in IMDM – Iscove's modified Dulbecco's medium with cytokines TPO, IL-6, SCF, FLT3-L + 30% FBS (Sigma, St. Louis, MO) and 1 mM valproic acid (sodium valproate, Sigma) at 37 °C in an atmosphere containing 5% carbon dioxide. The concentration of VPA was selected according to previous studies and corresponded to the clinically achieved blood level (Blaheta et al., 2005; Smetana et al, 2007). Regardless of the cultivation time, in control VPA-untreated cultures the number of cells ranged from 36 to $40 \times 10^4/1$ ml. In VPA-treated cultures the number of cells was significantly reduced and ranged from 20 to $25 \times 10^4/1$ ml (Table 1). Cytospins of cell cultures were prepared using a Universal 16R centrifuge, rotor 1624 (Hettich, Burladingen, Germany) for 5 min.

RNA of nucleolar bodies and cytoplasm was visualized in cytospins using methylene blue buffered with McIlvain's buffer at pH 5.3 without preceding fixation (Smetana et al., 1969; Busch and Smetana, 1970; Ochs, 1998). The acid pH and citric acid of the buffer prevented the loss of RNA from unfixed cells in cytospins that were not older than 24 h. The used method also facilitated distinction of the main nucleolar classes as well as measurement of the RNA density in myeloblasts and lymphocytes present in the studied cytospins (Figs. 1–5).

Micrographs were captured with a Camedia digital photo camera C.4040 ZOOM (Olympus, Tokyo, Japan) placed on a Jenalumar microscope (Zeiss, Jena, Germany) with double adapters to increase the magnification on the computer screen. The increased contrast of stained
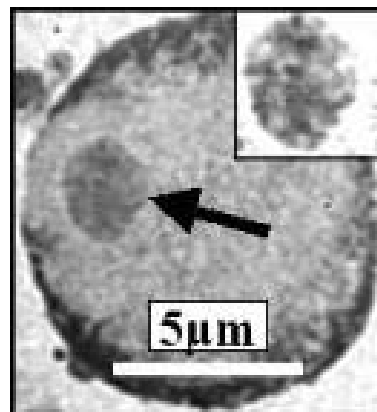

Fig. 1. VPA-untreated myeloblast with a large nucleolus (arrow) containing small light regions that correspond to the fibrillar centres in the electron microscope (insert). The bold black bar in this and following Figures represents 5 μm.
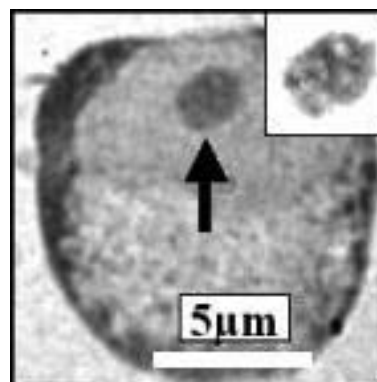

Fig. 2. VPA-treated cell with a single nucleolus (arrow) that is slightly smaller than that in Figure 1. The nucleolus in the enlarged insert contains small light regions similarly as those in the previous Figure.

Table 1. The number of cells/1 ml of cultivation medium and the incidence of leukaemic myeloblasts with the main nucleolar classes in VPA-untreated and -treated short-term cultures[a]

VPA	Time (h)	Cells × 10³/1 ml	Cells (%) with		
			active	resting nucleoli	inactive
0	24	380 ± 14^b	92.5 ± 6.0	1.7 ± 2.1	5.4 ± 6.4
+		$243 \pm 28*$	$55.6 \pm 9.6*$	$28.9 \pm 8.7*$	11.6 ± 8.8
0	48	336 ± 22	93.4 ± 2.6	2.7 ± 1.8	3.7 ± 2.1
+		$246 \pm 4*$	$69.9 \pm 5.9*$	$24.9 \pm 10.6^\#$	5.3 ± 4.6

Legend
[a] - Based on three cultures from different patients suffering from acute myeloid leukaemia
[b] - **Mean** and standard deviation
* - Significant difference from control cells using *t*-test (P < 0.006)
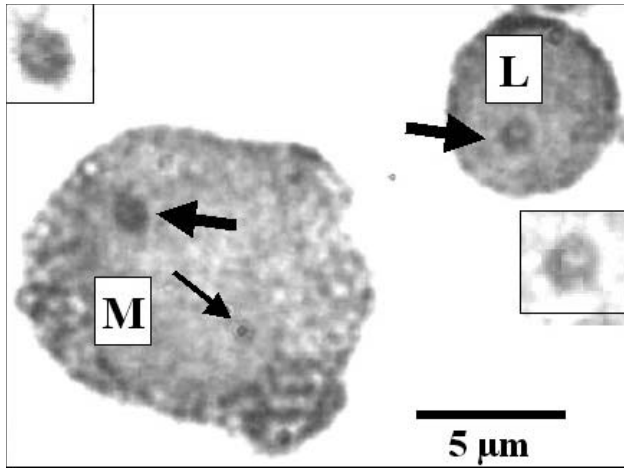# - Significant difference from control cells using *t*-test (P < 0.02)

Fig. 3. The myeloblast (M) contains both a ring-shaped nucleolus (thick arrow) and a micronucleolus (thin arrow). Mature lymphocyte (L) with a ring-shaped nucleolus (arrow). Ring-shaped nucleoli are also in inserts to better see the peripheral distribution of the nucleolar RNA. VPA treatment.
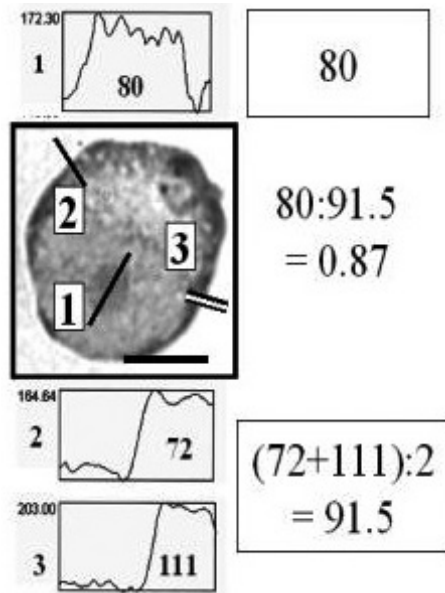

Fig. 4. The nucleolar (1) and cytoplasmic (2,3) RNA image density measurement (black lines in the micrograph) of a VPA-untreated myeloblast. The numbers on the left side of the density graphs correspond to measured lines. Numbers in density graphs represent arbitrary density units subtracted from the cell-surrounding background. The calculated nucleolar to cytoplasmic density ratio is on the right side of the micrograph. The bold black line in this and following Figure represents 5 μm.

Fig. 5. The nucleolar (1) and cytoplasmic RNA (2,3) image density measurement (black lines in the micrograph of a VPA-treated myeloblast). For other legend see the previous Figure.

erick, MD). The RNA image density reflecting the RNA concentration in nucleoli and cytoplasm was expressed in arbitrary density units calculated by subtracting the mean background density surrounding each measured cell from measured nucleolar or cytoplasmic densities (Smetana et al., 2006; Figs. 4, 5). The cytoplasmic as well as background densities were measured in two locations which exhibited the lowest and highest positivity. Such calculations and standardization of arbitrary density units facilitated the comparison of results in various peripheral regions of cytospins. The nucleolar to cytoplasmic RNA image density ratio for each cell was calculated by dividing mean nucleolar density by the cytoplasmic density (Smetana et al., 2006; Figs. 4, 5).

The results of measurements such as mean, standard deviation and significance of differences were evaluated using "Primer of Biostatistic Program, version 1" developed by S.A. Glantz (McGraw-Hill Inc., Toronto, Canada, 1968).

Results

The morphology of cultured cells in cytospins stained for RNA

Myeloblasts were characterized by a strong positivity for RNA in the cytoplasm and nucleolar bodies (Figs. 1–3). Some of these cells apparently started to differentiate and resembled the further stage – promyelocytes (Fig. 2). The nuclear chromatin, Golgi region, mitochondria and granules did not provide positive staining. As it was expected, in control cultures the incidence of intact cells without apparent alteration was very high, i.e. 90.5 ± 7.9 after 24 h and 80.6 ± 14.5 % after 48 h of cultivation. In contrast, cultures with VPA contained a

nucleolar bodies by image processing facilitated easy measurements of the nucleolar diameter using Quick Photoprogram (Olympus) to provide basic information on the nucleolar size.

The nucleolar and cytoplasmic RNA image density was measured after conversion of the captured coloured blue images to gray scale using the red channel (NIH Image Program, Scion for Windows, Scion Corp., Fred-
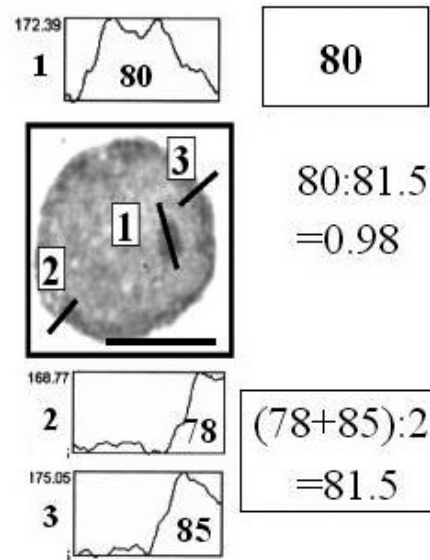
large percentage of markedly altered cells including apoptotic bodies and cell ghosts. The incidence of such cells was 33.8 ± 2 % after 24 h and 63.5 ± 17.2 % after 48 h of cultivation. It should be added that the studied cultures also contained mature lymphocytes with a characteristic morphology and smaller size than myeloblasts (Fig. 3). The incidence of these cells ranged from 4 to 11 % of cells present in cytospins of all VPA-untreated or -treated cultures. Most of these lymphocytes were mature and possessed a single resting ring-shaped nucleolus characteristic for these cells (Smetana, 2002, 2005, Fig. 3).

The nucleolar morphology and RNA distribution (Table 1)

The nucleolar morphology and RNA distribution in studied leukaemic blastic cells did not differ from previous descriptions (Smetana, 2002, 2005). Cultured control cells stained for RNA mostly possessed large nucleoli with more or less uniform distribution of RNA that frequently exhibited small light areas (Figs. 1, 2). These areas are known to correspond to fibrillar centres without RNA. It is generally accepted that the periphery of fibrillar centres are sites of RNA transcription (Wachtler and Stahl, 1993; Hozák, 1995; Raška et al., 2006). Large nucleoli with small light regions are considered to represent "active" nucleoli in respect of the nucleolar RNA transcription (Smetana et al., 2002, 2005). In cells cultured with VPA, the number of cells with "active" nucleoli significantly decreased and they were replaced by cells containing mainly "resting" ring-shaped nucleoli containing RNA in the nucleolar periphery or "inactive" micronucleoli (Fig. 3). The small size of micronucleoli does not make it possible to distinguish any particular distribution of RNA at the light microscopic level. Both ring-shaped nucleoli or micronucleoli are known to reflect the decrease or inhibition of the nucleolar RNA transcription (Schwarzacher and Wachtler, 1993; Wachtler and Stahl, 1993; Smetana, 2002, 2005) (Table 1).

The nucleolar size, nucleolar and cytoplasmic RNA image density (Table 2)

The nucleolar size in cultured leukaemic cells was expressed by the nucleolar diameter. The largest nucleolar diameter was found in control cells cultured for 24 h. The cultivation of control cells for 48 h produced its small reduction that, however, was not statistically significant. In contrast, the nucleolar diameter in VPA-treated cells was markedly reduced. The small differences of the nucleolar diameter between 24 and 48 h of cultivation with VPA were not statistically significant.

The values of the nucleolar and cytoplasmic RNA image density, i.e. nucleolar and cytoplasmic RNA concentrations, were similar and did not show significant differences between VPA-untreated and -treated myeloblasts. Therefore, the resulting calculated nucleolar to cytoplasmic RNA image density ratio ranged between 0.83 and 1.04 in both untreated and VPA-treated myeloblasts. Thus, the RNA concentration in nucleoli and cytoplasm was very stable regardless of the VPA treatment (Table 2).

Discussion

Similarly as in a previous study on Kasumi – myeloblasts (Smetana et al., 2007), in short-term cultures from patients suffering from acute myeloid leukaemia, VPA produced a heavy alteration. In addition, VPA also decreased the incidence of myeloblasts with "active" nucleoli and increased the percentage of cells with "resting" ring-shaped nucleoli and "inactive" micronucleoli. On this occasion it should be noted that ring-shaped nucleoli and micronucleoli reflect the decrease or inhibition of nucleolar biosynthetic activities in production of ribosomes (Smetana, 2002, 2005). It was also evident that VPA produced a significant reduction of the nucleolar diameter. Since the nucleolar size reduction is generally a sign of the decreased nucleolar biosynthetic activity and cell proliferation (Schwarzacher and Wachtler, 1993; Wachtler and Stahl, 1993; Smetana, 2005), VPA contributed to the decrease of both mentioned cell activities in addition to the nucleolar transformation (see above). On the other hand, since some myeloblasts did not exhibit the nucleolar transformation or nucleolar size reduction, such cells were apparently resistant to the VPA treatment.

Contrary to the VPA-induced transformation of nucleoli and decreased nucleolar size (see above), the nu-

Table 2. The nucleolar diameter, nucleolar RNA image density and nucleolar to cytoplasmic RNA image density ratio in VPA-untreated and -treated short-term cultures[a]

VPA	Time (h)	NoDm (μm)	NoDn	No/Cy Dn
0	24	**3.29** ± 0.30[b]	**69.9** ± 2.4	**0.93** ± 0.08
+		**1.88** ± 0.08*	**79.0** ± 12.4	**0.97** ± 0.06
0	48	**2.81** ± 0.21	**92.3** ± 7.5	**0.95** ± 0.04
+		**1.98** ± 0.15*	**79.4** ± 15.4	**1.04** ± 0.04

Legend
[a] - Based on three cultures from different patients suffering from acute myeloid leukaemia
Apoptotic cells or bodies and cell ghosts were not evaluated.
[b] - **Mean** and standard deviation
* - Significant difference from control cells using *t*-test (P < 0.006)
NoDm – nucleolar diameter, No – nucleolar, Cy – cytoplasmic, Dn – RNA image density

cleolar and cytoplasmic RNA concentration – expressed by the RNA image density – did not show significant changes. Thus, such marked difference between the decreased nucleolar diameter and constant RNA image density suggested that the RNA content in nucleolar bodies of VPA-treated leukaemic myeloblasts was apparently related to the nucleolar size. Such relationship was also noted previously in a variety of cells including neurocytes (Edström and Eichler, 1958). In addition, no differences of the RNA image density were noted between small and large nucleoli in early granulocytic progenitors as well as in peripheral monocytes (Smetana et al., 2006, 2008b).

Summarizing the above-presented results and discussion, it seems to be clear that the treatment of leukaemic myeloblasts with a HDACI, VPA, produces a distinct nucleolar alteration that is known to be related to the altered main nucleolar functions.

Acknowledgement

References

Blaheta, R. A., Michaelis, M., Driever, P. H., Cinatl, J. (2005) Evolving anticancer drug valproic acid: insight into mechanism of clinical studies. *Med. Res. Rev.* **25**, 383-393.

Boisvert, F. M., van Konigsbruggen, S., Navascués, J. A. I. (2007) The multifunctional nucleolus. *Nat. Rev. Mol. Cell Biol.* **8,** 574-585.

Busch, H., Smetana, K. (1970) *The Nucleolus.* Academic Press, New York.

Duenas-Gonzalez, A., Candelaria, M., Perez –Plascencia, C., Perez-Cardenas, E., de la Cruz-Hernandez, E., Herrera, L. A. (2008) Valproic acid as epigenetic cancer drug: preclinical, clinical and transcriptional effects on solid tumors. *Cancer Treat. Rev.* **34,** 206-222.

Edström, J. H., Eichner, D. (1958) Quantitative ribonuklein-säure-untersuchungen an den ganglienzellen des nucleus supraopticus der Albino-ratte under experimentellen bedigungen (kochsaltz-belastung). *Z. Zellforsch. mikrosk. anat.* **48,** 187-200.

Emanuele, S., Lauricella, M., Tesoriere, G. (2008) Histone deacetylase inhibitors: apoptotic effects and clinical implications. *Int. J. Oncol.* **33,** 637-646.

Eot-Houllier, G., Fulcrant, G., Magnaghi-Jaulin, L., Jaulin, C. (2009) Histone deacetylase inhibitors and genomic instability. *Cancer Lett.* **274,** 169-176.

Fakan, S., Puvion, E. (1980) The ultrastructural visualization of nucleolar and extranucleolar RNA synthesis and distribution. *Int. Rev. Cytol.* **65,** 255-299**.**

Han, D. M., Chen, X. O., Liang, R., Dong, B. X., Yin, W. (2006) Inhibition of proliferation and induction of apoptosis and differentiation of leukemic cell line by sodium valproate. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* **14,** 21-24.

Hozák, P. (1995) Catching RNA polymerase I in flagranti: ribosomal genes are transcribed in the dense fibrillar component of the nucleolus. *Exp. Cell Res.* **216**, 285-289.

Hrebackova, J., Hrabeta, J., Eckschager, T. (2010) Valproic acid in the complex therapy of maligant tumors. *Curr. Drug Targets* **11**, 361-379.

Olson, M. O., Dundr, M., Szebeni, A. (2000) The nucleolus: an old factory with unexpected capabilities. *Trends Cell Biol.* **10**, 189-196.

Pederson T. (1998). The plurifunctional nucleolus. *Nucleic Acids Res.* **26**, 3871-3876.

Ochs, R L. (1998) Methods used to study structure and function of the nucleolus. *Methods Cell Biol.* **53,** 303-301.

Papi, A., Ferreri, A. M., Rocchi, P., Guerra, F., Orlandi, M. (2010) Epigenetic modifiers as anticancer drugs: effectiveness of valproic acid in neural crest-derived tumor cells. *Anticancer Res.* **30,** 535-540.

Raška, I., Shaw, P. J., Cmarko, D. (2006) New insights into nucleolar architecture and activity. *Int. Rev. Cytol.* **255**, 177-235.

Santini, V., Gozzini, A., Ferrari, G. (2007) Histone deacetylase inhibitors: molecular and biological activity as a premise to clinical application. *Curr. Drug Metab.* **8**, 383-393.

Schwarzacher, H. G., Wachtler, F. (1993) The nucleolus. *Anat. Embryol.* **188**, 513-536.

Smetana, K., Lejnar, J., Potměšil, M. (1969) A further contribution to the demonstration of RNA and nucleoli of blood cells in smear preparations. *Folia Haematol.* **91**, 381-384.

Smetana K. (2002) Structural features in blood, leukemic, lymphoma and myeloma cells. *Eur. J. Histochem.* **46,** 125-132.

Smetana, K. (2005) Nucleoli in blood cells of hematologic malignancies (Structure, cytochemistry of nucleoli in leukemic, lymphoma and myeloma cells). In: *Trends in Leukaemia Research*, Romero, R. M. ed. Nova Science Publishers, Hauppage, New York, pp.155-179.

Smetana, K., Klamová, H., Mikulenková, D., Pluskalová, M., Hrkal Z. (2006) On the nucleolar size and density in human early granulocytic progenitors, myeloblasts. *Eur. J. Histochem.* **50**, 119-124.

Smetana, K., Zápotocký, M., Starková, J., Trka, J. (2007) Effect of histone deacetylase inhibitors on the cell nucleus and nucleolus of leukemic myeloblasts *in vitro* – a cytochemical study. *Acta Histochem.* **109**, 413-419.

Smetana, K., Zápotocký, M., Starková, J., Trka, J. (2008a) To the nucleolar density and size in apoptotic human leukemic myeloblasts produced *in vitro* by Trichostatin A. *Eur. J. Histochem.* **52**, 143-148.

Smetana, K., Jirásková, I., Otevřelová, P., Kalousek, I. (2008b) The RNA content of nucleolar bodies is related to their size – a cytochemical study on human monocytes and lymphocytes in blood smears and blood cytospins. *Folia Biol. (Praha)* **54,** 130-133.

Tan, J., Cang, S., Ma, Y., Liu, D. (2010) Novel histone deacetylase inhibitors in clinical trials as anti-cancer agents. *J. Hematol. Oncol.* **3**, 5.

Tschochner, H., Hurt, E. (2003) Pre-ribosomes on the road from the nucleolus to the cytoplasm. *Trends Cell Biol.* **13**, 255-263.

Wachtler, F., Stahl, A. (1993) The nucleolus: structural and functional interpretation. *Micron* **24**, 473-505.