Intracellular magnesium content decreases during mitochondria-mediated apoptosis induced by a new indole-derivative in human colon cancer cells

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Abstract. A newly synthesized indole-derivative is able to induce cytostatic and cytotoxic effects in the colon cancer cells HT29, effecting apoptosis by activation of an intrinsic pathway. Magnesium is involved in both cell growth and apoptosis even though its role in the latter process is not well defined. The aims of this work were: firstly, to verify if magnesium content is related to the proliferative rate in HT29 cells; secondly, to assess the involvement of the cation in mitochondria-mediated apoptosis triggered by the new antiproliferative molecule. The effects of the indole-derivative in treated cells included cell-cycle arrest in the G2/M phase, and apoptotic death confirmed by release of cytochrome c from the mitochondrial compartment. Moreover, we demonstrated that the basal content of magnesium in HT29 cells inversely correlates with cell saturation density. In addition, a decrease in both free and intracellular total magnesium concentration was observed along with the induced apoptosis. Taken together, these data suggest that magnesium participates in the complex signaling network of cell proliferation and apoptosis.

Key words: apoptosis, magnesium, indole-derivative, colon cancer

Apoptosis plays an essential role in the development and maintenance of tissue homeostasis; its deregulation results in a variety of diseases including cancer [1]. Dysfunction of the apoptotic pathways can not only promote tumorigenesis, but can also render cancer cells resistant to conventional anti-cancer agents, since the effect of chemotherapy and radiotherapy on cancer cells is mainly mediated through activation of apoptosis [1, 2]. Apoptosis is primarily executed by the caspases, cysteinyl-aspartate-specific proteases classified as initiators and effectors [3, 4]. They orchestrate apoptosis through the cleavage of numerous target proteins, ultimately leading to
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Figure 1. Structure of the indole-derivative, compound 5.

the phagocytic recognition and engulfment of the dying cell. The apoptotic process can be triggered by two main, alternative pathways, the extrinsic and the intrinsic pathways [5]. In the former, death receptor ligation causes the recruitment of adaptor molecules, that bind and activate the initiator caspase 8, which directly cleaves and activates the executioner caspases [6]. In the intrinsic pathway, mitochondrial outer membrane permeabilization leads to the release into the cytosol of pro-apoptotic proteins, such as cytochrome c. These proteins determine the activation of the initiator caspase 9, which, in turn, cleaves and activates the executioner caspases [7, 8].

In a previous study, we analyzed the cytotoxic activity of a family of 20 newly synthesized imidazothiazolymethylene-oxindole derivatives, which exhibited antiproliferative effects towards different cancer cell lines. Three of these compounds are active on colon carcinoma cells HT29 at nanomolar concentrations, similarly to the Vinca alkaloids. They too contain an indolic scaffold in their structure. In particular, the derivative named compound 5 by Andreani et al. [9] (figure 1), induced the maximal activation of the executioner caspases, together with an increased amount of the cell cycle inhibitor p21 protein. Moreover, we observed that compound 5 triggered apoptosis in HT29 cells with an increase of ROS production, Bax traslocation into mitochondria, and disruption of the mitochondrial membrane potential [9].

Growing evidence supports a role for magnesium in the apoptotic process [10]. ATP synthesis is regulated by the mitochondrial magnesium concentration [11], the latter being regulated via Mrs2, which is the Mg^{2+}-selective channel expressed in the inner mitochondrial membrane [12]. Moreover, it is reported that inner mitochondrial membrane depolarization induces the release of magnesium from mitochondria [13]. All this experimental evidence strongly supports the idea of a link between mitochondrial function and intracellular magnesium homeostasis.

The role of magnesium in the process of cell proliferation has been known for decades. According to Rubin’s “coordinate response theory”, magnesium influences the growth of eukaryotic cells in vitro through its requirement for protein and DNA synthesis [14-16]. Chien et al. observed magnesium mobilization in B cells undergoing Fas-initiated apoptosis: Fas-binding molecule expressed on the cell surface initiates multiple signalling pathways that result in apoptotic cell death and an increment in the cytosolic free Mg^{2+} concentration [17, 18]. Some authors suggested that the increase in magnesium concentration influences magnesium-dependent endonucleases activity, as well as cytochrome c release from mitochondria [19-21]. On the other hand, it has been demonstrated that exogenous peroxynitrite triggers apoptosis by inducing a decline in intracellular free Mg^{2+}, and that apoptosis can be reversed by adding magnesium to the cell medium [22].

It is important to clarify whether the variation in intracellular magnesium content occurring during the apoptotic process is a coincidental or causative event of the apoptotic cascade. Furthermore, besides the role in apoptosis, a second messenger role for magnesium during T-cell activation was recently demonstrated [23]. Although these signalling pathways pertain to different functions, the possibility that magnesium acts as an intracellular second messenger in the apoptotic scenario represents an intriguing hypothesis worthy of investigation. In this light, a first step is the assessment of the intracellular magnesium concentration during induction of apoptosis.

We evaluated the correlation between magnesium content and apoptosis in colon cancer cells treated with compound 5. The analysis were performed using atomic absorption spectroscopy (AAS) and flow cytometry, with a novel Diaza-Crown-Hydroxy-Quinoline (DCHQ) fluorescent chemosensor [24, 25], which allows the evaluation of intracellular total magnesium concentration in intact cells and in very small samples.
Materials and methods

Cells and treatment

The human HT29 colon adenocarcinoma cell line was maintained in RPMI 1640 medium (PAA, laboratories GmbH, Pashing, Austria) supplemented with 2mM L-glutamine (Sigma Aldrich St. Louis, MO, USA), 10% FBS (PAA, Laboratories GmbH, Pashing, Austria) at 37°C and 5% CO2. The cells were seeded in 64 cm² large dishes at the concentration of 2.5 × 10⁴/cm². Cell densities were determined at 24 h intervals by counting cells with a hemocytometer after trypsinization. Compound 5 was dissolved in DMSO to a concentration of 10 mM. Cells were allowed to grow for one day (at 25% of confluence) and treated with 500 nM compound 5 for 24 h.

Cell cycle analysis

Cell cycle analysis was performed according to Nüssé et al. [26]. Briefly, about 2 × 10⁶ cells were centrifuged and 1 mL of solution I (0.584 g/L NaCl, 1.139 mg/L sodium citrate, 10 mg/L RNase, 0.3 mL/L Nonidet P-40 0.03% v/v) was added to the cell pellet. After about 30 min, 1 mL of solution II (100 mg/L propidium iodide (PI) 0.25 M sucrose, 1.5% citric acid) was added to complete the fixing and staining. The cell suspension was agitated and kept at 4°C until flow cytometry measurements. Flow cytometric analyses were carried out using an Epics-XL Beckman Coulter equipped with a 15 mW Argon ion laser source and analyzed with ModFit (Verity Software House, USA) software.

Analysis of cytochrome c release

Cells (1 × 10⁶) were harvested and treated with 100 μL digitonin (50 μg/mL in PBS with 100 mM KCl) for 5 min on ice (until >95% were permeabilized as assessed by trypan blue exclusion). Cells were fixed in paraformaldehyde (4% in PBS) for 20 min at room temperature, washed three times in PBS and incubated in blocking buffer (3% BSA, 0.05% saponin in PBS) for 1 h. The cells were incubated overnight at 4°C with 1:200 anti-cytochrome c monoclonal antibody (BD Pharmingen, San Diego, CA, USA) in blocking buffer, washed three times and incubated for 1 h at room temperature in 1:200 FITC labelled secondary antibody (Sigma Aldrich, Milan, Italy) in blocking buffer [27]. The cells were then analyzed by flow cytometry, and cytochrome release was quantified using WinDMI 2.8 software.

Measurement of free and total magnesium concentration by flow cytometry

The detection of total magnesium was carried out using the new fluorescent probe DCHQ5, dissolved to a final concentration of 1.37 mM in DMSO [25]. Before the staining, HT29 cells were washed twice in PBS without Mg2+ (8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L Na2HPO4, 0.2 g/L KH2PO4, pH 7.2), and suspended at a final concentration of 1 × 10⁶ cells/mL. The cells were incubated for 15 min in the dark at 37°C with 5 μM fluorophore and then analyzed. Free Mg2+ levels were determined in cells loaded with Mag-Fluo4-AM dye. After washing, HT29 cells were suspended in medium without Mg2+ and then incubated for 30 min at 37°C with 0.8 μM fluorophore. The choice of the medium without magnesium was the result of wanting staining conditions similar to those used for DCHQ5. Finally, the samples were centrifuged and resuspended in fresh medium prior to analysis. Dead cells were excluded by PI incorporation.

Measurement of total magnesium concentration by AAS

HT29 cells were harvested, counted and centrifuged at 200 g for 10 min. The pellet was washed twice with PBS, then resuspended in a solution of 1 M HNO₃, at a final concentration of 6.7 × 10⁵ cells/mL and left at 4°C overnight. Before the analysis, the acid digested was centrifuged and the supernatant used for the magnesium quantification. Calibration standards (4 μM, 12 μM and 24 μM) were prepared with Mg(NO₃)₂ dissolved in ultrapure water. Samples were analyzed with a Beckman Coulter spectrometer equipped with a magnesium-specific lamp and the flame was fired by air/acylene.
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Figure 2. Effect of a 24-h treatment with compound 5 (500 nM) on the HT29 cell cycle, detected by flow cytometric analysis of DNA content. The figure depicts the results obtained in one experiment representative of three.

Data are presented as mean ± SD. When SDs are not provided, data represent a typical experiment repeated at least three times with similar results.

Results

Compound 5 causes perturbation of the cell cycle and cytochrome c release in HT29 cells.

We evaluated the effect of the indole derivative on the cell cycle. As shown in figure 2, the treatment induced cell-cycle perturbation, which was characterized by increased G2/M peak (53% ± 3 versus 15% ± 4 of control) and decreased G0/G1 peak (15% ± 2 versus 57% ± 4 of control). In parallel, we observed a marked reduction in cytochrome c content in treated cells (figure 3).

Magnesium content decreases with cell density and apoptosis in HT29 cells

We determined the magnesium content as a function of cell density and after treatment with compound 5. AAS results showed that the intracellular total magnesium concentration correlates inversely with cell saturation density (figure 4A), and that the apoptosis induced by compound 5 decreases the magnesium content (figure 4B).

Free and total magnesium content decreases during mitochondrial-mediated apoptosis

We performed cytofluorimetric analyses to assess the cellular free and total magnesium concentration in HT-29 cells after 24 h of treatment with compound 5. Free cytosolic Mg2+ was investigated using the probe MagFluo4-AM. Cytofluorimetric measurements revealed a consistent decline in Mg2+ (about 40% in treated cells with respect to controls, figure 5A). Furthermore, we measured the intracellular total magnesium by using the newly synthesized DCHQ5 fluorescent probe [23].

Figure 3. Flow cytometric analysis of cytochrome c release in HT29 cells after 24 h of treatment with compound 5. The plot shows the results obtained in one experiment representative of three.
Figure 4. Quantitative determination of HT29 magnesium content measured by AAS: A) at different saturation density values; B) after treatment with compound 5 (500 nM) for 24 h. The differences between all samples reported are statistically significant (p<0.05 Student’s t-test).

Figure 5. Flow cytometric analysis of intracellular free (A) and total (B) magnesium content in HT29 cells after 24 h of treatment with compound 5 (500 nM). Typical experiments repeated at least three times with similar results.

Figure 5B shows a considerable decrease in fluorescence, confirming the previously described loss of total magnesium in treated samples.

Discussion

In this study, we have further detailed the cytotoxic activity of the indole-derivative compound 5, which induces apoptosis in different cancer cell lines. Our results integrate the published data [9], and better characterize the pro-apoptotic activity of the indole-derivative in HT29 cells, demonstrating that an intrinsic pathway is involved. In fact, the treated cells, mainly blocked in the G2/M phase of the cell cycle, showed a consistent release of cytochrome c, providing clear-cut evidence of mitochondrial involvement in the apoptosis induced by compound 5.

Apoptosis is not only a genetically-controlled mechanism essential for the maintenance of tissue homeostasis, development and elimination of
unwanted cells (such as cancer cells), but also a commonly accepted anti-neoplastic mechanism for chemotherapeutic agents [28, 29]. Cations are important in apoptosis since they regulate protein activation in the different signal transduction pathways related to it [30]. Particular emphasis has been placed on the influence of the Ca²⁺ ion, because it is considered the most important intracellular messenger. More recently, the influence of Mg²⁺ ion on apoptosis is gaining increasing attention, although the literature available on the link between magnesium and apoptosis is still scarce and often ambiguous. It was reported that Fas–induced B cell apoptosis requires an increase in free cytosolic Mg²⁺ as an early event [18]. On the other hand, a decline in free cytosolic Mg²⁺ was observed during peroxynitrite-induced apoptosis in rat aortic smooth muscle cells [22]. In order to elucidate the role of magnesium in apoptosis, we investigated the variations in intracellular magnesium content during mitochondria-mediated apoptosis after treatment with the new indole-derivative. Since the mitochondrion is the intracellular compartment where magnesium is stored [11, 24, 31], the hypothesis of its involvement in the intrinsic pathway is particularly intriguing.

Firstly, to assess whether magnesium, in HT29 cells, has a controlling role in cell proliferation, we analyzed the intracellular magnesium content as a function of the proliferative state, using samples at different cell densities. It is well known that the growth rate of this tumour cell line decreases along with confluence [32, 33]. Our data demonstrated an inverse correlation between the ion concentration and cell saturation density, in agreement with results reported in literature regarding normal epithelial cells [34]. The expression of transient receptor potential melastatin 7 (TRPM7) channels, which represent the major magnesium-uptake mechanism in mammalian cells, has not been investigated in the HT29 cell line. Since TRPM7 is over-expressed in a variety of human carcinoma cells, where it regulates proliferation and invasion [35], it would be interesting to investigate its expression in the HT29 cell line.

Secondly, we investigated whether there were variations in intracellular magnesium concentrations during compound 5-induced apoptosis: we observed a significant decrease in cation content in treated samples. In recent years, it has been demonstrated that sizable fluxes of magnesium can cross the plasma membrane in either direction following hormonal and non–hormonal stimuli, resulting in major changes in total and, to a lesser extent, free Mg²⁺ content [31]. Analogously to other divalent cations, magnesium may be both free and bound to biological ligands (nucleic acids, ATP, proteins and phospholipids), and it is well known that the two forms undergo different and independent regulatory mechanisms. On these assumptions, we considered it appropriate to discriminate between the two forms of the ion, in order to monitor their variations during mitochondria-mediated apoptosis. The analysis was carried out with flow cytometry, using the commercial probe MagFluo4-AM (Kd(Mg²⁺) = 4.7 mM) to detect ionized magnesium, and by the newly synthesized DCHQ5 (Kd(Mg²⁺) = 8.3 μM) to detect total magnesium. DCHQ5 belongs to a family of fluorescent hydroxyquinoline derivatives showing a remarkable affinity and specificity for magnesium, higher than the other commercially available probes. In particular, this probe allows a versatile flow-cytometric semi-quantitative determination of total magnesium concentrations in whole cells, being excited in UV and the visible field [25, 36]. Therefore, this analytical approach allowed a multiparametric analysis, confirming in living cells the quantitative data obtained by the reference method AAS.

In conclusion, the analysis performed in treated cells revealed a loss of total intracellular magnesium content, detected by both AAS and cytometric analysis, as well as of the ionized form during the mitochondria-activated apoptotic process.

The picture on the role of magnesium in apoptosis is far from complete: further experimental data are needed to elucidate the involvement of this cation in the apoptotic process. Our data provide clear indications that the role of magnesium in programmed cell death is multifaceted and is much more significant than previously believed.

**Disclosure**

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References


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