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Can metal replacement increase the antitumor activity of azurin? (*in vitro* study)

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Abstract

Azurin is a member of blue-copper proteins family which induces apoptosis in cancer cells. In the present work, azurin (Az) has been modified via replacement of copper at active site with cobalt and nickel. FTIR revealed that its secondary structure is practically indistinguishable from that of its derivatives. UV spectroscopy and DSC results revealed that native form of Az is more stable than Co- and Ni forms. SRB was used to elucidate their possible anti-tumor effect on cancer cell lines from breast and colon cells (MCF-7 and HCT116, respectively). To our best knowledge, this is the first time metal replacement of Az has been used with cell line. It appears that this process is the predominant reason for the effectiveness of Az. Experimental results showed that the anti-tumor activity of Cu- and Co Az (at concentration ≤ 25 μg/L) is higher for colon-relative to breast cells, while that for Ni-Az is higher for both cells (at low concentration, 5 μg/L). Hence, according to our findings it was revealed that Ni-Az form has the most extensive anti-antitumor effect on breast-and colon cancer cells. This highlights that Ni may be bona fide copper ion impersonator *in vivo*.

Introduction

Currently cancer represents one of the greatest burdens of our society and will remain a serious and challenging major public health problem in the future years. Each year about 12.7 million people are diagnosed with cancer, and approximately 7.6 million die from it, clearly demonstrating the magnitude of this disease in human population. Breast cancer is the second most common cancer worldwide, with 1.4 million cases being diagnosed annually, and the first cause of cancer-related death among women (458,000 deaths/year) [1]. Current cancer treatments rely on surgery, chemo-and radiotherapy, or even hormone therapy, in the case of breast cancer. However, these therapies can reveal serious and systemic side-effects in patient's health due to its high toxicity and lack of cancerous tissue specificity [2]. Additionally not every patient responds efficiently to chemotherapy or other treatments, since cancer cells can undergo micro-evolution and rapidly render cancer cells resistant to drug therapy [3]. Therefore, today we face new challenges regarding cancer treatment and cancer patients, especially those which do not respond to conventional therapies, demand for new, more efficient and selective drugs or therapies to fight this disease.

Newer and alternative anti-cancer therapies are under development and are inevitable to reach more successful results against cancer than the conventional radio-and chemotherapies. The use of microorganisms and its derived products, especially proteins, to treat this disease are being broadly explored and would probably overcome the flaws unspecific cancer treatments.

For the first time in 1956, the *Pseudomonas aeruginosa* bacterium was reported to contain a blue protein [4]. They proposed the name 'azurin' (Az) for this class of proteins due to purplish shade of blue of the copper ions present in their structure. It functions as an electron carrier [5] and eradicates the host defense system by encouraging apoptosis in phagocyte cells [6]. However, a new and interesting role regarding Az was revealed in 2000, when Zaborina, *et al.* [7] reported Az cytotoxic and apoptosis-inducing activities towards murine macrophage cell line J774. Later it was shown that Az can also trigger apoptosis and lead to significant cytotoxicity in different human tumor cell lines as breast cancer (MCF-7), melanoma (UISO-Mel-2) and osteosarcoma (U2OS) cells [8,9]. Interestingly Az exhibits preferentially selectivity against tumor cell lines, showing much less cytotoxic and apoptotic effects towards normal cell lines [10].

Since Az has been reported to be a potential anticancer protein against breast cancer cell lines, researchers are searching for novel methods to enhance its production. Ramachandran, *et al.* [11] focused on enhanced Az synthesis from four different strains of *P. aeruginos*a with apparent homogeneity and stability by adding both $CuSo_4$ and KNO_3 in the culture medium.

Substitution of the naturally occurring metals in metallo-proteins can be considered as a kind of site directed mutation. Metal substitutions in proteins have previously been successfully used to probe structural and electronic states of metal sites in these proteins. Metal ions, such as Ni (II) and Co (II), have then been used as a replacement of the native metal ion in many blue copper proteins.

In the present work, Az has been modified is via replacement of copper at the active site with other metal ions. We have chosen to investigate the interaction of cobalt and nickel metals with *P. aeruginosa* Az. There are several reasons for studying the binding of Ni (II) and Co (II) to Az. The atomic number of Co, Ni and Cu is 27, 28 and 29

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respectively. They belong to the first transition series. The energies of the 3d and 4s orbitals in the neutral atoms are quite similar, and their configurations are 3dn4s2 except for Cu (3d104s1), which is attributed to the stability of the filled d shell. In addition, cobalt containing compounds recently attracted considerable interest as systemic anticancer agents [12], while nickel (II) is vital for living beings [13] and it has been reported that Ni has apoptogenic capabilities [14].

On such a background we use UV absorption spectroscopy (UV) and denaturation experiments, monitored by differential scanning calorimetry (DSC), to investigate the effect of metal substitution on Az stability. Then cell line (SRB) was used to describe and elucidate their possible anti-tumour and cytotoxic effects on cancer cell lines from breast and colon (MCF-7 and HCT116, respectively). To our best knowledge, this is the first time metal replacement of Az has been used with cell line.

Experimental

Materials and samples preparation

All the chemicals used in this work were of analytical reagent grade and obtained from Sigma Chemical Co.

Holo Az (with cofactor) from *Pseudomonas Aeruginosa* was obtained as previously described [15]. Apo-Az (without cofactor) was prepared by a cyanide treatment of the holo form according to the procedure described elsewhere by dialysis against 0.1 M KCN in 20 mM tris-HCl, pH=8, for 14 days at room temperature [16]. After cyanide treatment, the protein was dissolved in 10 mM tris-base buffer at pH=8. The ionic strength was adjusted at 0.1 M by sodium chloride. Incubation of apo-Az with a three-fold excess of Cu (II) resulted in a rapid reconstitution of the holo-form. The concentration of apo-Az was determined by measuring the UV absorbance of the protein solution at 280 nm.

The Az metallo-derivatives were prepared by adding a 5-10 folds excess of metal ions (cobalt (II) sulfate and nickel (II) sulfate) to the apo-protein solution. The metal uptake was allowed to take place overnight at 4 °C. Reconstitution with Ni (II) gave a pale green solution, while that with Co (II) resulted in a yellow solution.

Ultraviolet absorption spectroscopy (UV)

UV optical spectra were recorded at room temperature (25°C) in a1cm cell in a Unicam UV 2-300 UV/visible spectrometer, in the wavelength region 200-800 nm. The reported UV spectra were the average of 3 scans. The error in the reading was less than 8% (standard curve estimation). The blank employed for baseline subtraction consisted of tris-base buffer.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were recorded using a double beam IR spectrometer of type Jasco-460 (FTIR plus, Japan). Each spectrum is recorded over wave number range 4000-400 cm^{-1}. Az samples were mixed intimately with finely powdered KBr and the mixture is squeezed in a press to about 1000 atmospheres. Under these conditions the KBr becomes glassy and forms a thin translucent disc in which the finally ground sample is suspended and we kept the tris-base buffer as a background medium and performed all measurements at room temperature.

Differential scanning calorimetry (DSC)

DSC analysis was performed by a Shimadzu DSC-50 calorimeter. Samples were scanned from room temperature to 100°C, with a heating rate 1°C/min. The protein (10 μg/ml) was dissolved in mM tris-base buffer at pH =8. The ionic strength was adjusted at 0.1 by sodium chloride. The thermogram represents the enthalpy flow rate in mW on the Y-axis and temperature on the X-axis.

Measurement of potential cytotoxicity by SRB assay

Cell lines used for testing *in vitro* cytotoxicity included MCF-7 and HCT116 derived from human breast and colon carcinoma cell lines respectively were supplied from National Cancer Institute, Egypt. All of the procedures concerning the cell culture maintenance and treatment were carried out in a "Napaco" Laminar flow cabinet.

The potential cytotoxicity of Az was tested [17]. Cells were plated on 96-multiwell plate (104cells/well) for 24 hours before treatment with Az to allow attachment of cell to the wall of the plate. Different concentrations of Az under test (0, 5, 12, 25 and 50μg/mL) were added to the cell monolayer triplicate wells which prepared for each individual dose. Monolayer cells were incubated with Az for 48 hours at 37°C and in atmosphere of 5% CO2. After 48 hours, cells were fixed, washed and stained with SRB stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. The relation between surviving fraction and Az concentration is plotted to get the survival curve of each tumor cell line after the specified Az.

Statistics

The data processing included the variance ANOVA test with $P<0.05$ taken as significance level, using Microsoft EXCEL for PC.

Results and discussion

Spectroscopic characterization of Cu (II)-, Ni (II)-and Co (II) azurin

The purity of Az was checked by PAGE and UV-visible spectroscopy. Purified Cu (II)-Az had an A_{625}/A_{280} ratio of 0.54-0.55. After the cyanide treatment, on introducing Cu (II) into the apo-Az, the characteristic blue color of native Az rapidly developed, and the absorbance ratio (A_{625}/A_{280}) of the reconstituted protein Cu (II) Az was 0.5 or greater. It has been shown that under comparable conditions, Cu (I1) was taken up by apo-Az much more rapidly than Ni (II) or Co (I1) [18]. On introducing Ni (I1) into a solution of apo-Az, a yellow color developed, but much less rapidly than the coloration induced by Cu (II), while a faint bluish color appeared when Co (I1) was used.

We have verified that Ni and Co can metallate apo-Az using absorption spectroscopy which demonstrates that these metals effectively metallates *P. aeruginosa* apo-azurin in solution as presented in Figure 1. Az has strong charge-transfer absorption with maximum absorbance at around 625 nm due to the bond between Cu and Cys-112 [19]. The absorption band probes the oxidation state of the copper ion and other alterations around it [20]. As shown in Figure 1, Ni- and Co-Az have intense peak near 391 and 308 nm respectively, this is in agreement with Czernuszewicz, *et al.* [21]. The charge-transfer absorption bands of the nickel (II) and copper (II) derivatives appear in the visible region, while that of cobalt (II) derivative appear in the near-UV region [22]. These shifts in peaks positions is due to ligand-to-metal charge transfer transitions, which are expected to increase in energy as one moves from right to left across the periodic table as a result of the acceptor d-orbitals shifting to higher energy.

FTIR analysis

The functional groups of Az were studied using FTIR spectrum. Az provides a convenient model to study infrared spectra of proteins forming characteristic β-barrel structures. Figure 2 shows the FTIR

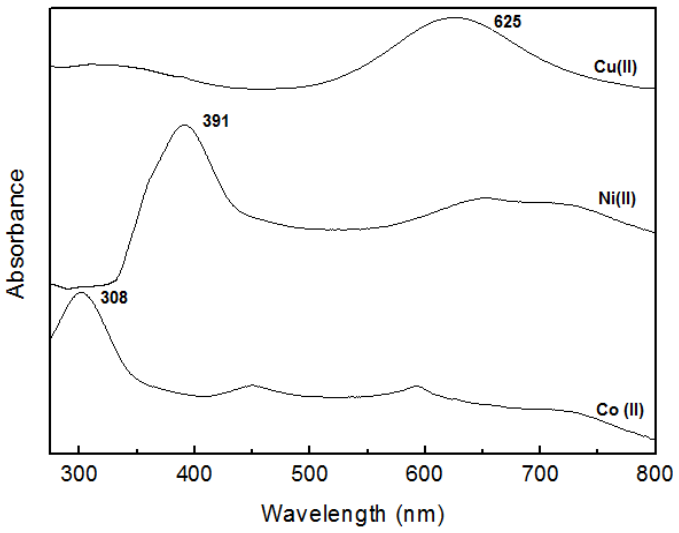

Figure 1. Room-temperature absorption spectra of (a) native *P. aeruginosa azurin* and its (b) Ni (II)-and (c) Co (II) substituted derivatives

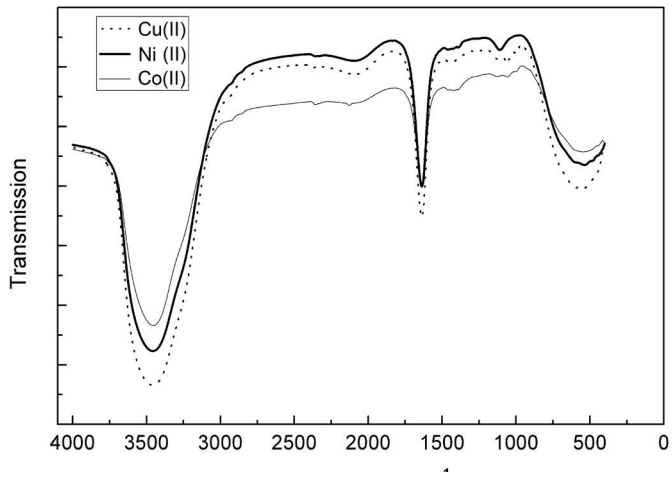

Figure 2. FTIR spectra of native *P. aeruginosa azurin* (dot line) and it's Co (II)-(thick line) and Ni (II)-(thin line) substituted derivatives recorded at room temperature

spectrum of wild-type (Cu-containing) *P. aeruginosa* Az and its Co (II)-and Ni (II) substituted metallo-derivatives recorded at room temperature.

Metal ion variation allowed a possibility to assess the extent to which protein structural constraints govern conformational balance in blue copper centers. Moreover, it has been suggested that replacement of native Cu(II) by other metal ions should have minimal effects on the metal binding site because of the existence of an extensive network of hydrogen bonds and other interactions that make the copper site more rigid and thereby a more ordered part of the structure [23]. As shown in Figure 2, the secondary structure of native Az is practically indistinguishable from that of cobalt and nickel derivatives. This similarity does not, of course, preclude localized differences in the vicinity of the active site. Crystal structures of apo-and holo Az have shown that the overall three-dimensional structure is identical with and without a metal (copper or zinc) cofactor [24].

As presented in Figure 2, FTIR spectra exhibit characteristic bands for α-helix, β-sheet, β-turn, and random coil conformations in the amide I (1700–1600 cm^{-1}) and amide II (1560–1500 cm^{-1}) regions [25]. Among these regions, amide I (due to the C=O stretch vibrations of the peptide linkages) is more sensitive to protein secondary structures. It is well established that α-helical conformation has an amide I and II (mainly from in-plane N-H bending and from the C-N stretching vibration) bands between 1657 and 1650 cm^{-1}, and between 1550 and 1540 cm^{-1}, respectively [26]. The β-sheet has an amide I and II bands between 1635 and 1615 cm^{-1}, and between 1535 and 1520 cm^{-1}, respectively [27].

Our findings indicated the presence of the amide I band by the maximum peak around 1650 cm^{-1} region for the three proteins, which is a characteristic feature of β-structure, and arises primarily because of the stretching vibration of the main chain of carbonyl groups in the protein backbone coupled with the in-plane N-H bending and C-N stretching modes [28]. The presence of the amide II band was indicated by the peak around 1495 cm^{-1}, 1462 cm^{-1} and 1425 cm^{-1} for native-, Ni- and Co-Az respectively, which arises because of the C-N stretching as well as the C-N-H bending motions [11].

Unlike amide I and II, the N-H stretching vibration (3310–3270 cm^{-1}) of peptide bond (–CO-NH-) is insensitive to the conformation of the polypeptide backbone as it is exclusively localized on the NH group. But the frequency of N-H stretching depends on the strength of the hydrogen bond. In the present study, the characteristic frequency of hydrogen bonded N-H has been observed at 3457 cm^{-1}, 3456 cm^{-1} and 3453 cm^{-1} for Cu (II)-, Ni (II)- and Co (II) Az, respectively, confirming the folded conformation of Az. It folds into three-dimensional structure known as β-barrel structure arranged in double wound Greek key topology [29].

FTIR and amide I band results revealed that nickel- and cobalt Az spectra closely resembles that for native Az. Baker [30] showed that the structure of apo-Az is practically identical to that of the holo-protein. The copper ligands are held in place owing to the existence of a tight cluster of hydrogen bonds as well as van der Waals interactions, and the surrounding structure is the most tightly constrained, least flexible part of the whole molecule. This has led to the idea that the protein imposes fairly rigid coordination geometry on the copper site [31] which is mainly kept even when the metal is substituted. The Az metal site is tailored for copper binding since it offers three strong equatorial ligands and two weakly bound axial ligands.

Moratal, *et al.* [32] showed that Az can bind different metals without drastic conformational changes, but the rigidity of the metal site cannot prevent slight reorganizations, including some distortions at the polypeptide backbone and side-chain atoms in the proximity of the co-ordination center. To rationalize the different binding modes of Cu-, Co- and Ni, their particular characteristics can be considered. In conclusion, the overall structure of Az is not modified by the metal exchange and the only differences with regard to the native copper Az occurred in the metal site region. These variations affect principally the axial ligands. Nickel co-ordinates more strongly to the carbonyl oxygen of Gly45 while its distance to the Met121 S4 enlarges up to 0.330 nm. The resulting metal center structure can be described as distorted tetrahedral. It might not be expected that such relatively small differences would result in large differences in their interactions with proteins, but differences in electronic structure along metals play their part too, and the coordination geometries of host proteins can be exquisitely discriminating. Small differences in distances, especially if they involve charged groups, may well change the energy of interaction sufficiently to make a large difference to biological outcome. Chen, *et al.* [33] showed that even subtle changes in the structure of metal complexes can result in dramatic changes in their physicochemical and thus biological properties.

Differential scanning calorimetry

It seemed of interest to find out whether the replacement of copper affects the structural stability of Az. In metallo-proteins, the understanding of the contribution of the metal ion to the thermodynamic stability of the protein structure can be investigated through studying of DSC. With this aim, denaturation experiments were carried out on native-, nickel- and cobalt-Az. Denaturation was achieved by heating and was monitored by DSC.

A common feature of all the proteins analyzed, is the irreversibility of the calorimetric transition, as indicated by the absence of endothermic effects on a second scan of the samples (data not shown). Both the Tm value and the irreversible character of the thermal transition of all proteins are in agreement with the DSC data previously reported [34,35].

All Az samples showed a single calorimetric peak (data not shown) while the comparison of the three curves evidences some differences. The main differences of the Ni and Co derivatives with respect to the Cu form are: (i) a lower thermal stability in terms of denaturation temperature as the temperature of maximum heat absorption (Tmax) is about 62°C and 67°C for Ni- and Co-Az respectively, whereas for Cu protein the same parameter is about 74°C. (ii) lower symmetry of the curve.

The relative instability of the nickel complex is readily understood in terms of simple ligand-field theory which shows that the loss in ligand-field stabilization energy as a result of the change from octahedral to tetrahedral symmetry is greatest in the case of d^8 ions [36].

The corresponding values of the temperature of maximum heat capacity (Tmax) and the experimental enthalpy values (ΔH) values as a function of the scan rates are listed in Table 1.

The enthalpy data appear to give an indication of the relative stabilities of the various metallated forms. In particular, it suggests that Cu stabilizes the folded form of the protein more than Co and that either of these derivatives is more stable than the Ni derivative (Table1). This is in agreement with Engeseth and McMillin [34] who showed that Cu -azurin had the highest denaturation enthalpy. Leckner, *et al.* [37] showed that copper ion is coordinated to the polypeptide chain in five positions and these interactions contribute to the Az stability.

The reduced stability of the Co- and Ni-Az is evident from both the downward shift of transition temperature from native to denaturated state of the protein as well as from the decrease of ΔH value which may be explained by change in the metal center which is better described as a distorted tetrahedron, instead of trigonal bipyramidal as in the Cu – Az [21]. These results suggest that the metal substitution is less effective in stabilizing Az. Bonander, *et al.* [38] showed that the presence of both a disulfide bridge and a copper atom enhance the structural stability of native Az.

Role of metals in toxicity of azurin *in vitro*

Experiments involving *P. aeruginosa* bacterium and cancer cells have demonstrated that Az secretion occurs mainly in presence of cancer cells in the medium, whereas in the absence of them very little secretion of this protein was verified. These findings point out the possible existence of a sensing mechanism in bacteria that could lead to Az secretion in the presence of cancer cells, which they could sense as a threat or competitor to their own growth [39]. The possibility of these proteins to act on different pathways, like controlling apoptosis and cell invasion processes make them a unique weapon to be explored in the future years.

Table 1. Comparison between maximum heat capacity and experimental enthalpy values for Cu-, Co - and Ni-azurin obtained by using DSC at scanning rate1°C/min.

Sample	Maximum heat capacity (Tm) °C	Experimental enthalpy values (ΔH) (KJ/g)
Cu-Az	74.39	648
Co- Az	66.99	556.44
Ni-Az	62.37	476.66

Despite a wealth of studies on Az–metal interactions, there has been less focus on the effect of this process on Az toxicity. It emerges that the roles of metal ions in Az toxicity can vary dramatically. As it was mentioned above, Az by itself enters cancer cells preferentially. Accordingly, if it is conjugated to Co- or Ni, it may be more specifically localized at the tumor site and exerts its apoptosis-inducing function, harming much less normal cells. We may take better advantage of the Az anti-tumor activity, making its action more selective, more efficient and enhance Az cytotoxicity with minimal side effects on normal tissues by metal replacement. Maret [40] showed that metal substitution is an important mechanism in pathophysiology and toxicology, because functions of metalloproteins depend on the type of metal ion.

The second main objective in this work was to study the effect of Az modification (with Co and Ni) on its anti-tumor effect (using SRB assay) on cancer cell lines from human breast and colon cells (MCF-7 and HCT116, respectively). Az (from *Pseudomonas aeruginosa*) was used as positive control in order to compare their relative effects, since its cytotoxicity toward tumor cells was already described.

In-vitro cytotoxicity of Az samples was obtained using SRB treatment against MCF7and HCT116 cells treated with Cu-, Co- and Ni -Az at different concentrations (0, 5, 12, 25, 50 μg/ml) for 24 hours and the data is reported in Figures 3a, 3b and 3c respectively.

As presented in Figure 3, the proportion of Az required for growth inhibition varies according to the cell line and the concentration of Az used. Figure 3a showed that Cu-Az has greater significant ($P<0.05$) cytotoxic effect on HCT116- than MCF-7 cell lines at low concentrations (5 and μg/ml) but by increasing concentration it shows more cytotoxic effect towards MCF-7 cells (25 and 50 μg/ml). Data revealed that 50 μg/ml (the highest used concentration) is the optimum concentration for Cu- Az to be more effective as an antitumor agent for both HCT116- and MCF-7 cell lines.

Goto, *et al.* [41] demonstrated that the redox activity is not important for Az cytotoxicity. The fact that apo-Az (without copper) demonstrated high ROS levels and cytotoxicity, comparable to wt Az (with copper), appears to indicate that it is not copper but the Az protein that is important for its interaction with the tumor-suppressor p53 protein.

Results revealed that cobalt derivative shows a small cytotoxic effect towards both cell lines (Figure 3b). It has the best significant cytotoxic effect on HCT116- and MCF-7 cell line at concentrations of 12 and 50 μg/ml respectively.

One of the most encouraging observations made in our study is that Ni-Az with very low concentration (5 μg/ml) gives the same anti-tumor activity on HCT116 cell line as that recorded for Cu-Az but with tenfold increase in concentration (50 μg/ml). While it has greater significant ($P<0.05$) cytotoxicity effect on MCF-7 cell line than that for Cu-Az for all concentration < 50 μg /ml (Figure 3c).

Abd-Elzaher, *et al.* [42] determined the anticancer properties of ferrocenyl complexes with Co (II), Ni (II), Cu (II), and Zn (II) against MCF-7. The highest antitumor activity was found for Zn and

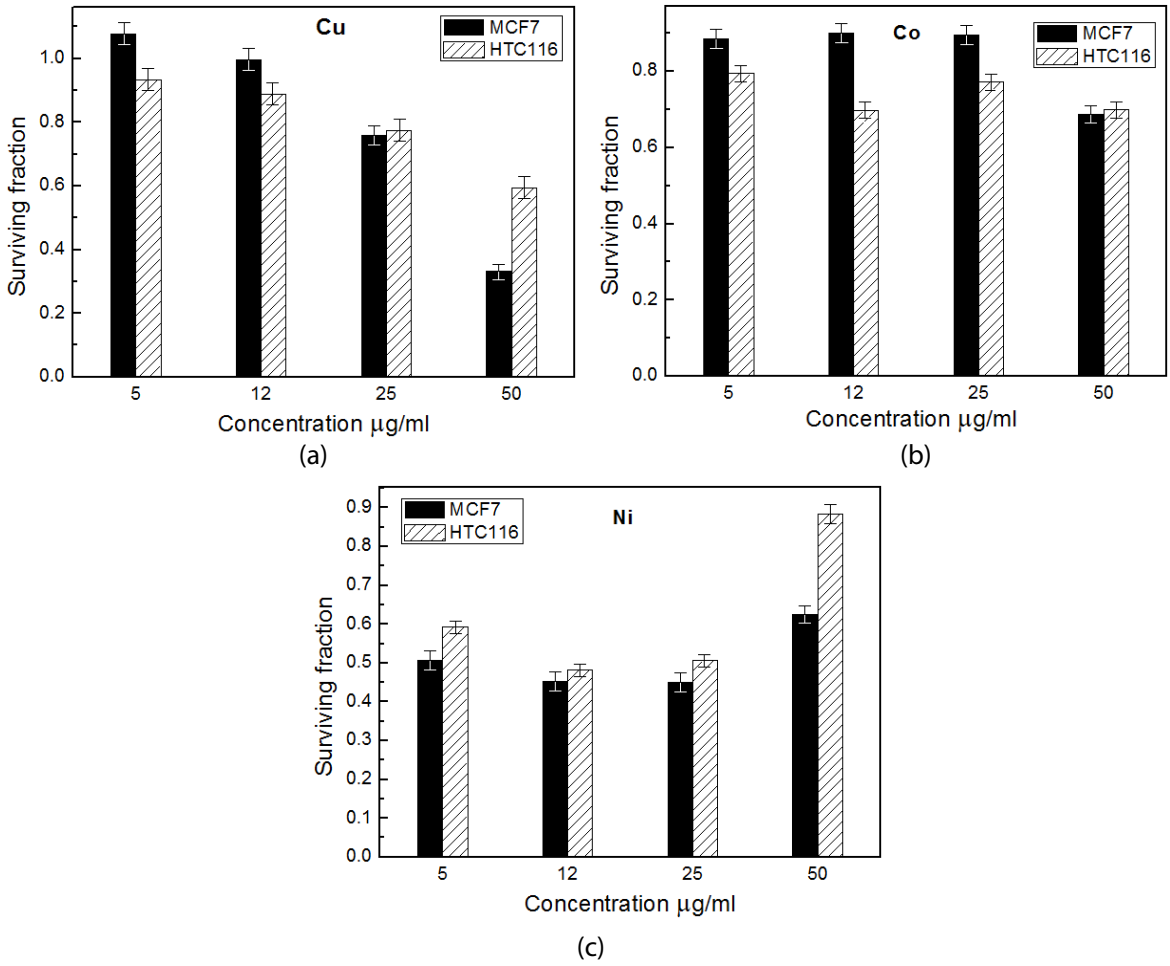

Figure 3. Cytotoxic effect of Cu-(a), Co -(b) and Ni- azurin(c) on MCF7 and HCT116 cell lines at different concentrations using SRB assay

Ni complexes, which resulted in the highest superoxide dismutase (SOD) activity and hydrogen peroxide (H_2O_2) and low activities of catalase (CAT) and glutathione peroxidase (GSH-Px) as well as glutathione (GSH) level. They concluded that the antitumor effect of these complexes is exerted by production of H_2O_2. Similarly, Gomes-Junior, *et al.* [43] showed that nickel elicits a fast antioxidant response in *Coffea arabica* cells; $NiCl_2$ induced the antioxidant defences by the rapid increase of the activity of all of the major antioxidant enzymes such as SOD and CAT. This is in agreement with Bienvenu, *et al.* [44] who showed that most chemotherapeutic agents cause cells to over generate ROS and thus are capable of inducing apoptosis and necrosis by causing oxidative damage to DNA, proteins and lipids.

On the other hand, many articles have reported DNA binding and anticancer potentials of nickel complexes owing to good affinity of Ni(II) for DNA and some DNA binding proteins [45,46].

Afrasiabi, *et al.* [47] showed that complexation with nickel ion in semicarbazone derivative increases the inhibitory action on MCF-7 cell proliferation. Similar effect was observed upon complexation of other thiosemicarbazones with nickel (II) ion [48]. The enhancement of antiproliferation activity by metal complexes is related to an increase in the lipophilicity so they can penetrate into the cells more easily [49].

Taken together, it appears that this combination of all the above mentioned factors act in an additive or synergistic mode.

To our best knowledge, this is the first time metal replacement of Az has been used (with cell line) in an attempt to find derivatives with greater potency and high toxicity than the exiting Az. Data revealed that this process is the predominant reason for the effectiveness of Az and enhancement of its activity towards tumor cells. This is in a full agreement with Samie, *et al.* [50] who showed that nickel complex is a potent anti-cancer agent by simultaneous induction of intrinsic and extrinsic pathways of apoptosis as well as cell cycle arrest in colon and breast cancer cell lines.

The data presented in Figures 3a, 3b and 3c are represented in Figure 4a and Figure 4b to clarify the effect of metal substitution on anti-tumor activity of Az on cancer and colon cells respectively.

Figures 4a and 4b showed that Ni- Az can be considered as the most promising potent antitumor protein relative to the others. This is in agreement with Aliasa, *et al.* [51] who showed the nickel (II) complex has higher cytotoxicity than Co (II) due to high polarity and large size of this metal in complex ion. Since the high polarity gives covalent character in ionic bond to the complexes and made easy to enter in the cell. Obviously, small concentration of the used heavy metal will be more safe for healthy cells. This gives Ni –Az greater advantage relative to the other forms. Results also revealed that Ni have low cytotoxic effect in high concentrations than other concentrations i.e. low and middle concentrations, this is common in toxicology and called Hormesis which means that the compound showed inhibition effect at low concentrations but not at high concentrations [51].

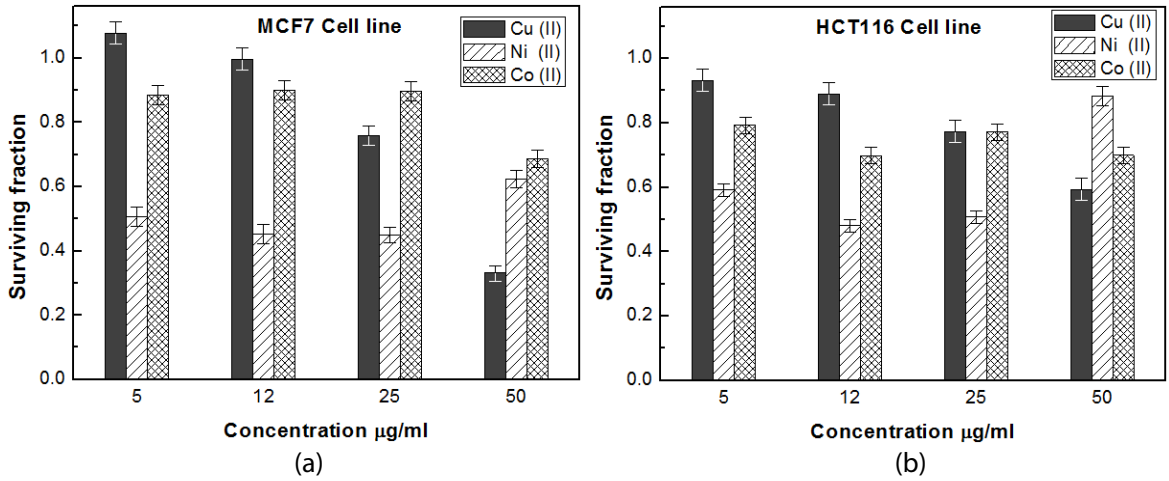

Figure 4. Cytotoxic effect of Cu-, Co-and Ni- azurin on MCF7 cell line (a) and HCT116 cell line (b) at different concentrations using SRB assay

According to these results, Cu and Co derivatives may be defective in complex formation with p53, generates low levels of ROS (reactive oxygen species) and lacks appreciable cytotoxicity towards breast and colon cell lines in comparison with Ni. Many factors may be responsible in the activity of these complexes in pharmacological field like size of metal, charge distribution, geometry shape, ionic character, and polarity [52].

Obviously this observation merits further studies to truly understand the nature and significance of this replacement, but we suggest that it highlights that Ni may be bona fide copper ion impersonator *in vivo*. The next step in this field (in progress) is to relate the *in vitro* observations to *in vivo* conditions. The situation inside of cells is more complex than a dilute buffer system and one should use caution before extrapolating *in vitro* conclusions to the *in vivo* scenario.

Conclusion

We have verified that cobalt and nickel can metallate apo-Az using multiple biophysical techniques. Our idea of metal replacement was not only for enhancing Az antitumor activity, but to reveal the differences of Az's stability and the secondary structure. One of the encouraging observations made in our study is that Ni- Az form has the most extensive anti-antitumor effect on breast- and colon cancer cell line.

Conflict of interest

The authors report no conflict of interest.

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