BioMetals **17:** 443–450, 2004.

Mini-Review

Effects of AZT on cellular iron homeostasis

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Received 14 November 2003; accepted 8 January 2004; Published online: April 2004

Key words: 3′-azido-3′-deoxythymidine (AZT), iron, transferrin receptor (TfR), glycosylation, chelatable

Abstract

3′-azido-3′-deoxythymidine (AZT), the first chemotherapeutic drug approved by FDA for treatment of HIV-infected patients and still used in combination therapy, has been shown to induce, upon prolonged exposure, severe bone marrow toxicity manifested as anemia, neutropenia and siderosis. These toxic effects are caused by inhibition of heme synthesis and, as a consequence, transferrin receptor (TfR) number appears increased and so iron taken up by cells. Since iron overload can promote the frequency and severity of many infections, siderosis is viewed as a further burden for AIDS patients. We have previously demonstrated that AZT-treated K562 cells showed an increase of the number of TfRs located on the surface of the plasma membrane without affecting their biosynthesis, but slowing down their endocytotic pathway. In spite of the higher number of receptors on the plasma-membrane of AZT-treated cells, intracellular accumulation of iron showed a similar level in control and in drug-exposed cells. The chelating ability of AZT and of its phosphorylated derivatives, both in an acellular system and in K562 cells, was also checked. The results demonstrated that AZT and AZTMP were uneffective as iron chelators, while AZTTP displayed a significant capacity to remove iron from transferrin (Tf). Our results suggest that AZT may be not directly involved in the iron overloading observed upon its prolonged use in AIDS therapy. The iron accumulation found in these patients is instead caused by other unknown mechanisms that need further studies to be clarified.

Abbreviations: AIDS – acquired immunodeficiency syndrome; ALAS – α-aminolevulinic acid synthase; AMT – 3′-amino-3′-deoxythymidine; AZT – 3′-azido-3′-deoxythymidine; AZTMP – 3′-azido-3′-deoxythymidine monophosphate; AZTTP – 3′-azido-3′-deoxythymidine triphosphate; CNS – central nervous system; GATA-1 and NFE-2 – erythroid-specific transcription factors; HIV – human immunodeficiency virus; IREs – iron responsive elements; LIP – intracellular labile iron pool; MDR – multiple drug resistance; SDS-PAGE – sodium dodecylsulfate poliacrylamide gel electroforesis; Tf – transferrin; TfR – transferrin receptor.

AZT-induced bone marrow toxicity and siderosis

3′-azido-3′-deoxythymidine (AZT, zidovudine) is one of the first chemotherapeutic agents approved for the first-line therapy against human immunodeficiency virus (HIV) infection and it is still used in combination with protease inhibitors. Unfortunately the clinical benefits of AZT treatment in AIDS patients are often reduced, especially after prolonged therapy, by the appearance of drug-resistance (Yarchoan *et al.* 1989, Yusa *et al.* 1990, Avramis *et al.* 1993). In addition, treatment with AZT is limited by its toxic effects to bone marrow cells, manifested as anemia and neutropenia (Richmann *et al.* 1987). Studies performed in bone marrow cells from AZT-injected rats demonstrate that heme synthesis is impaired (Lutton *et al.*

1990). In particular, the rate-limiting enzyme for heme biosynthesis α-aminolevulinic acid synthase (ALAS) is suppressed, which could account for some of the inhibition of hematopoiesis observed in the AZT-treated animals. Previous investigations have indicated that the mRNA half-life for ALAS is approximately 45 min, and AZT toxicity could be mediated in part through a direct reduction in the half-life of mRNA for ALAS or other enzymes and subsequent heme synthesis (Abraham *et al.* 1985). As a consequence, it may be expected that AZT treatment upregulate the synthesis of transferrin receptors because when heme binds to the iron responsive element binding protein, it inhibits transferrin receptor synthesis and when intracellular heme is depleted, transferrin receptor number appears increased (Pelicci *et al.* 1982, Ward *et al.* 1984, Lin *et al.* 1991). A rise of transferrin receptors will increase the amount of iron taken up by cells (Jacopetta *et al.* 1982, Pelosi-Testa *et al.* 1986). Since iron excess can enhance the frequency and severity of many infections, siderosis could be a particular problem for HIV-infected patients. Nevertheless, the net effect of AZT is to increase tissue iron and this effect is potentially detrimental. Presumably this drug would be still more effective if the iron loading were prevented (Pollack & Weaver 1993).

Anaemia in HIV-infected patients

The hematologic complications of HIV-infected patients include anaemia, neutropenia, lymphopenia and thrombocytopenia. Early in the HIV pandemic it was recognized that anaemia was a prognostic marker of future disease progression or death, independent of CD4 number and viral load. Additionally, anaemia impacts negatively the quality of life, in subjects with HIV infection, most commonly due to its association with fatigue (Ludwig & Strasser 2001). Recovery from anaemia reduces the risk of disease progression to approximately the same level as observed among patients who have never had anaemia (Sullivan *et al.* 1998). Anaemia can be caused by a range of mechanisms including infections, neoplasms, dietary deficiencies, blood loss and clinical management of patients (Volberding 2000). Histologically, bone marrow hypoproliferation and dysplasia are most commonly observed. Inhibition of erythrocyte progenitor cell differentiation in the bone marrow by HIV or *Mycobacterium avium* complex (MAC) infection may reduce red blood cell production. AZT causes macrocytosis and has a broad myelosuppressive effect both *in vitro* and *in vivo.* Chronic disease is usually associated with anaemia often in conjunction with low erythropoietin levels. Additionally, other cytochines such as tumor necrosis factor a and transforming growth factor b may appear upregulated in HIV infection, contributing to ineffective red cell proliferation. Deficiencies in iron, Vitamin B12 or folate are unusual in HIV-infected patients, at least in the developed world. Iron deficiency is commonly related to blood loss and vitamin deficiencies to malabsorption secondary to severe gastrointestinal infections. The management of anaemia typically includes correction of the underlying cause(s) and blood transfusion or administration of erythropoietin. However, it seems that blood transfusions may directly accelerate HIV disease progression through activation of HIV expression and possibly by triggering immunosuppression (Groopman 1997). Supplementation with iron should also be done with caution, as iron could be harmful to individuals with HIV infection, being associated in some studies with increased progression risk (Gordeuk *et al.* 2001). Recombinant human erythropoietin is an effective means of improving haemoglobin and reducing transfusion requirements in HIV-infected patients who show low endogenous erythropoietin levels (Henry *et al.* 1992).

AZT Inhibits erythroid-specific transcription factors

It has been reported that AZT and its *in vivo* metabolite 3′-amino-3′-deoxythymidine (AMT), affect regulation of specific genes, suggesting that inhibition of erythroid differentiation may also play a pivotal role in AZT-induced anemia (Weider & Sommadossi 1990, 1991). In particular, AZT and AMT inhibited hemoglobin synthesis and globin mRNA transcription rate in butyric acid-induced human K562 erythroleukemia cells. Such inhibitory effects are associated with a decrease in the binding levels of the two known erythroid-specific transcription factors, GATA-1 and NFE-2, to their consensus sequences. A marked fall in GATA-1 and NFE-2 mRNA was in fact observed 24 h after AZT exposure and was evident up to 72 h (Bridges *et al.* 1996). These *in vitro* studies are in agreement with *in vivo* investigations where administration of AZT to cats infected with feline leukemia virus and in a murine model of AIDS is associated with progressive anemia (Chow *et al.* 1991). In addi-

tion, AZT has been reported to inhibit erythropoietin receptor expression in murine bone marrow progenitor cells (Gogu *et al.* 1992). There are GATA-1 binding sites in the promoter region of the GATA-1 gene (Nicolis *et al.* 1991), globin genes (Martin *et al.* 1989, Nicolis *et al.* 1989) and the erythropoietin receptor genes (Chiba *et al.* 1991). Thus, a decrease in the activity of erythroid-specific transcription factors, leading to a blockage in erythroid differentiation, may play an important role in AZT-induced anemia.

AZT Induction of MDR1-P-glycoprotein

The innate or acquired expression of an efficient drug transport system is the main route by which tumor cells can actively proliferate in the presence of several cytotoxic drugs (Germann *et al.* 1993). Such multiple drug resistance (MDR) is one of the most challenging topics in Medicine since it represents a severe impairement to successful chemotherapy of tumours or infectious diseases by pathogenic microorganisms (Childs & Ling 1994). In human cell lines, this form of MDR is usually mediated by the over-expression of MDR1 gene-encoded class-I-P-glycoprotein (P-glycoprotein) which acts as an ATP-driven drug-efflux pump, decreasing intracellular anticancer drugs bioavailability (Kartner *et al.* 1983, Schinkel & Borst 1991). Experiments carried out to determine whether AZT is able to induce MDR1 expression in tumor cells would be of great medical relevance considering the high risk of contracting cancers by AIDS patients (Hermans & Clumeck 1995). Literature data demonstrate that treatment of tumor cells with AZT induces expression of MDR1-P-glycoprotein and that this efflux system interferes with the cytotoxic efficacy of anti-neoplastic drugs such as vinblastine (Signoretti *et al.* 1997). Moreover, flow cytometry studies show that a short-term exposure (24 h) of the human T-lymphoblastoid cells to AZT causes a homogeneous expression of P-glycoprotein in the cell population. This phenomenon seems to represent a general feature of the human lymphoblastoid cell lineage since other cell types, such as Jurkat and MOLT, respond to AZT treatment with an increased expression of the MDR1 gene (Chaudary & Roninson 1993). The *in vitro* experimental evidence that in various cell systems AZT induces P-glycoprotein expression may be of clinical relevance. In fact, under chemotherapy treatment, this event could favour the selection of genetic variants with highest levels of drug-resistance.

Antiretroviral activity and pharmacokinetics of AZT

Several papers report that AZT, upon entering the infected cells either passively via nonfacilitated diffusion (Zimmerman *et al.* 1987) or actively through specific nucleobase transporters (Domin *et al.* 1988), is efficiently converted to its monophosphorylated derivative (AZTMP) by cellular nucleoside kinases and subsequently, to a much lower extent, to di- and triphosphates (AZTDP and AZTTP) by nucleotide kinases (Furman *et al.* 1986, Balzarini *et al.* 1988). The latter compound, AZTTP, is the active form that interrupt the viral DNA chain elongation (Cheng *et al.* 1987, Kedar *et al.* 1990), but a major role has also been ascribed to AZT by itself and to AZTMP regarding the cytostatic and cytotoxic effects exhibited by this drug on infected cells (Cretton *et al.* 1991, Tornevik *et al.* 1995). By contrast, there is no significant toxicity toward healthy uninfected cells because of the relative insensitivity of eukaryotic polymerases, a feature that strongly favoured the use of AZT in anti-HIV therapy (Waquar *et al.* 1984, Furman *et al.* 1986). On the other hand, a prolonged exposure of lymphoblastoid cell lines to progressively increasing concentrations of the drug, has been shown to induce the expression of AZT-specific cellular resistance not involving necessarily an enhanced expression of the P-glycoprotein (Dianzani *et al.* 1994). A decreased activity (10–20 fold) of thymidine kinase (TK) has been observed in these drug-resistant cells (Nyce *et al.* 1993). This enzyme, responsible for the conversion of AZT to AZTMP, represents an essential step for further activation of the drug to AZTTP. The decreased activity of thymidine kinase in AZT-resistant cells has been interpreted on the basis of an AZT-induced hypermethylation, leading then to a lowered expression of the TK gene (Wu *et al.* 1995, Lucarelli *et al.* 1996). Therefore, a prolonged exposure to the drug may in principle select cell variants expressing alternative (or combined) mechanisms to avoid or reduce AZT-induced harmful side effects, like: i) altered permeation of AZT across the cell membrane; ii) drug extrusion by efflux systems; iii) reduced AZT phosphorylation and iv) increased nucleotidase-mediated AZTMP hydrolysis. These mechanisms are expected to exert different effects on AZT and AZTMP levels in either AZT-sensitive or in its resistant cell variant. An additional limitation to the continuous administration of this drug as a chemotherapeutic agent for HIV-infected patients, is due to the onset of delayed

myopathy which can appear irrespective of the duration of treatment and of the dosage levels (Gill *et al.* 1987, Dalakas *et al.* 1990). An interesting aspect of the *in vivo* pharmacology of AZT, not observed with most other clinically used dideoxynucleoside analogues, is its extensive conversion to and excretion as the biologically inactive 5′-O-glucuronide (Resetar & Spector 1989, Good *et al.* 1990). Another metabolic product of AZT is the corresponding amino compound 3′-amino-3′-deoxythymidine (AMT), produced by a NADPH-requiring reaction (Cretton *et al.* 1991). This agent, whose cytotoxic effects were initially investigated several years ago by Prusoff and his co-workers, exhibits some five-fold greater toxicity toward hemopoietic progenitor cells than does its parent compound AZT (Chen *et al.* 1984), and thus may account, at least in part, for the bone marrow suppression frequently seen after long-term administration of AZT at relatively high dose-levels.

Iron metabolism in eukaryotic cells

Physiologically, iron delivery to mammalian cells is mediated by the iron carrier transferrin (Tf), which initially binds to a specific cell surface receptor (TfR), undergoes endocytosis and, upon a short intracellular stage, it is lastly extruded (Kornfeld & Mellman 1989). It has been demonstrated that in human erythroleukemia cells (K562), the overall pathway duration is approximately 16 min (Young & Bomford 1994). Several studies have shown that the iron supply for the majority of cells is almost essentially via the TfR, and Tf is thus necessary for *in vitro* cell growth and proliferation (Melefors & Hentze 1993, Breuer *et al.* 1995). Although there is a close correlation between cell proliferation and TfR expression in many cell types (Trowbridge & Omary 1981), there are a number of non-proliferating cells, such as maturing erythroid cells or placental trophoblast cells, in which the high iron requirement is afforded by increased expression of TfR (Jandl & Katz 1963, Seligman *et al.* 1979). In these cellular systems, the altered function of TfR can directly modulate iron uptake. In fact, by changing either the rate of receptor biosynthesis and/or degradation or the affinity for the ligand, or the rate of endocytosis and/or exocytosis with a shift in the location of the receptors within the cell, so as to render them more or less available to the ligand without altering the total number of receptor molecules, the receptor function can be modulated (Schonhorn *et al.* 1995). On the basis of increasing clinical reports and *in vitro* studies, a close correlation between AZT treatment of HIV-positive patients and both the development of anaemia and iron overload have been described in the last years (Goldin *et al.* 1993, Boelaert *et al.* 1996, Salhi *et al.* 1998). Further observations suggest that in AZT-treated mice, an increase of non-heme iron load occurs both in the liver and in macrophages, the increment being higher in hepatocytes, regardless of the intensity and duration of the treatment. As the main conclusions the investigators hypothesized that TfR up-regulation could be attributed to the inhibition of heme synthesis (Pollack & Weaver 1993). In addition, it has been also demonstrated that intracellular iron levels affect the amount of TfR mRNA by controlling its life-time in the cytosol (Owen & Kühn 1987, Casey *et al.* 1988, Müllner *et al.* 1989).

Recent advances

In the light of the above reported studies we recently started to investigate, in an *in vitro* cell system, the effects of AZT on some features of the iron supply and regulation still not fully elucidated. To this purpose, human erythroleukemia cells (K562) were exposed for increasing times to different AZT concentrations, the best results being obtained in cells treated with 40 μM drug for 24 h. In particular, the TfR binding capacity, the rate of TfR endo-exocytosis and the iron uptake were studied. As far as the binding experiments concerns, K562 cells treated for 24 h with 40 μM AZT exhibited an increase of the number of TfR located on the cytoplasmic membrane while no variation of the apparent association constant was detected (D'Alessandro *et al.* 1999). To clarify if this increased number of total TfR_s in AZT-treated cells was due to a rise of the receptor protein biosynthesis or if the observed up-regulation was the result of a redistribution of receptors, we first determined the total amount (namely, intracellular plus cell surface) of TfR in control and AZT-treated cells. Immunoblot of both control and drug-exposed cell lysates, exhibited a single band with almost the same intensity. Moreover, ^{14}C-leucine metabolically labeled cells showed no significant difference in the biosynthesis of the receptors in AZT-treated cells as compared to control cells. On the contrary, both the SDS-PAGE analysis of cell surface TfR and immunoprecipitation of the metabolically labeled cell surface TfR displayed an overall increase of the receptor amount in drug-exposed cells (D'Alessandro

et al. 1999). Regarding endocytosis and reciclying of TfR, a significant slowing down of the endocytotic pathway was detected in AZT-treated cells. In fact, the value of the rate constant for the internalization of the ligand-receptor complexes showed a marked decrease, $t_{1/2}$ being 2.15 min in control vs. 3.75 min in drug-treated cells (D'Alessandro *et al.* 1999). Intracellular accumulation of iron in control or AZT-treated cells showed a very similar pattern regardless of the drug concentration assayed. In particular, the delivery rate of the radioactive iron associated with cells during the first 20 min treatment did not change significantly in the presence or in the absence of AZT, in spite of the higher number of binding receptors on the surface of the treated cells (D'Alessandro *et al.* 1999). It has to be underlined that all the above reported effects appeared evident after 24 h, which is approximately the normal time required by K562 for duplication, and initiated to be observed at 5 μM AZT, that is in the range of the plasma concentration reached during clinical treatment of HIV-infected subjects (Langtry & Campoli-Richards 1989). Our finding that AZT did not significantly affect the iron uptake via TfR, confirmed the hypothesis that the action of this drug on the iron metabolism is probably a multi-factorial process, leading to the conclusion that the iron amount taken up through TfR, by itself, does not account for the iron overload. On the other hand, our observations, in agreement with other reports, prompted us to hypothesize that the AZT-induced altered glycosylation in K562 cells might also affect TfR exposure and functionality, influencing the early steps of endosome formation and slowing down the endocytotic rate (Hall *et al.* 1994, Yan *et al.* 1995). In fact, we recently found that AZT inhibits glycoprotein sialylation in K562 cells and TfR is indeed a glycoprotein (D'Andrea *et al.* 1999). In particular, we focused our interest on the distribution of both protein glycans and some enzymatic activities involved in the glycan processing in AZT-treated K562 cells. After total sialic acid determination and sialoproteins visualization, a set of digoxigenated lectins was used to evidentiate the presence of specific terminally linked glycan residues on cell homogenates. The cell samples were also assayed for their β,(1 $\rightarrow$ 4) galactosyltransferase, β-galactosidase as well as the main sialyltransferase activities. Our results show that cells exposure to AZT caused a reduced silalylation, while β,(1$\rightarrow$4) galactosyltransferase and β-galactosidase activities exhibited a significant increase. By contrast, among the different sialyltransferases assayed, no significant differences were found between control and AZT-treated K562 cells, except the α,(2 $\rightarrow$ 8) sialyltransferase activity that markedly decreased in drug-exposed cells (D'Andrea *et al.* 2001, 2003).

Conclusions and perspectives

Clinical reports concerning AZT-treated patients that develop siderosis are mainly referred to subjects who are multiply transfused during the treatment of anemia (Lindley *et al.* 1989, Goldin *et al.* 1993), and a strict correlation between the amount of blood transfused and the degree of iron overload has been also described (Lindley *et al.* 1989). The rapid sequestration of iron from the chelatable pool is of particular importance in situations where cells are exposed to a sudden iron load. It is well established that oxidative damage caused by tissue ischemia/reperfusion can be significantly reduced by iron chelators. The basis of this preventive effect is believed to be the chelation of iron released from storage compartments, which in combination with the sudden oxidative burst following reperfusion, induces a rapid accumulation of oxygen radicals (Sussman & Bulkley 1990, Ryan & Aust 1992). Iron-related (chelator-inhibitable) oxidative injury has also been observed in a variety of pathological conditions involving heart, liver and CNS. Thus, an endogenous mechanism for rapid resequestration of excess cytoplasmic iron would minimize oxidative damage, similarly to the effect of externally added chelators. The efficiency of this mechanism could be an important determinant of the capacity of cells to recover from or counterbalance potential damage from transient iron loads (Brever *et al.* 1996). In an attempt to further elucidate the machanism(s) underlying AZT-induced siderosis, we have extended our previous observations investigating the chelating capacity of AZT and its phosphorylated derivatives either in solution and in K562 cells. To this purpose the composition and dynamic of the low-Mr iron pool (LIP) in cultured cells and the pattern of double labeled ^{125}I-^{59}Fe-transferrin uptake in AZT-treated and untreated K562 cells have been studied. These cells in logaritmic phase of growth, in RPMI medium supplemented with 10% FCS, exhibited a steady state level of 350 nM LIP (Brever *et al.* 1996). AZTTP and TTP were found quite effective in removing iron from Tf, at pH 6.1, although their efficiency was approximately 40%, as compared to ATP that is believed to be the main metabolite, *in vivo*, able to trap the chelatable iron pool

(Weaver & Pollack 1989, Weaver *et al.* 1990). On the contrary, AZT and AZTMP resulted practically uneffective. These data indicate that the thymidine moiety of AZT is not implicated, 'per se', in the iron removal from Tf. However, it has to be noticed that our data were obtained at pH 6.1; when checked at pH 7.4 at the same concentrations, all the compounds assayed, ATP included, were almost unable to remove iron from Tf. On the other hand, despite the finding that AZTTP displayed some iron-chelating capacity in solution, its very low intracellular concentration (around 0.5–1 μM) should scarcely affect iron levels (Kreutzer & Rockstrom 1997). On the basis of these observations, a significant direct effect of AZT and of its phosphorylated derivatives on the composition and dynamics of the low-Mr iron pool *in vivo* can be reasonably discarded. Taken together, our data seem to indicate that, although it has the potential to affect, at least in part, the Tf-mediated iron uptake in mammalian cells, AZT exposure does not lead to a marked increase of intracellular iron under our experimental conditions (D'Alessandro *et al.* 1999, 2000). These results suggest that AZT, as such, might be not directly involved in the iron overloading observed upon its prolonged use in AIDS therapy (Goldin *et al.* 1993), but it is likely to cause accumulation of excess iron in various tissues by other unknown mechanisms which need further studies to be clarified. In this regard, it should be recalled that AZT metabolism is highly dependent from the cell species (Balzarini *et al.* 1988, Di Vito *et al.* 1997). In patients with iron overload, deposition of excess iron occurs in almost all tissues; nevertheless the bulk of iron is detected mainly in reticuloendothelial cells, but it is also found in the spleen, in the liver, in the bone marrow and in parenchymal tissues, e.g. hepatocytes, endocrine system's cells and myocard (Hershko *et al.* 1998). Moreover, the source of iron and the proportions of iron retained in ferritin stores and recycled into the circulation from the reticuloendothelial or parenchymal cells differs remarkably. Although reticuloendothelial cells are the primary site of siderosis in conditions wherein iron overload is caused by multiple blood transfusions (Hershko *et al.* 1998), considerable redistribution of the metal in other body districts and cellular types may occur subsequently. It will be, therefore, of great interest to investigate whether AZT-treatment *in vitro* of these cell lines may induce an intracellular iron excess.

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