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Triazene Compounds in the Treatment of Acute Myeloid Leukemia: A Short Review and a Case Report

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Abstract: Acute myeloid leukemia (AML) is a highly lethal disease, especially in old patients. Chemoresistance and the absence of host immune responses against autochthonous malignancy play a major role in the poor prognosis of AML. The triazene compounds Dacarbazine and Temozolomide are monofunctional alkylators that donate methyl groups to many sites in DNA, including the O^6-position of guanine producing O^6-methylguanine (O^6-MeG). If not repaired, O^6-MeG frequently mispairs with thymine during DNA duplication. O^6-MeG:T mismatches can be recognized by the mismatch repair (MMR) system which activates a cascade of molecular events leading to cell cycle arrest and cell death. If MMR is defective, cells continue to divide and GC → AT transition mutations occur. In preclinical models, such mutations can lead to the appearance of abnormal proteins containing non-self peptides ("chemical xenogenization" CX) that can be recognized by host cell-mediated immunity. Repair of O^6-MeG is achieved by the DNA repair protein, O^6-methylguanine-DNA methyltransferase (MGMT), which removes the methyl adduct in an autoinactivating stoichiometric reaction. High MGMT levels attenuate the pharmacodynamic effects of triazenes. In the last few years, triazenes, alone or with MGMT inhibitors, have been tested in AML. In view of their potential activity as CX inducers, triazenes could offer the additional advantage of host anti-leukemia immune responses. The present paper describes several studies of leukemia treatment with triazenes and a case of acute refractory leukemia with massive skin infiltration by malignant cells. Treatment with Temozolomide and Lomeguatrib, a potent MGMT inhibitor, produced a huge, although transient, blastolysis and complete disappearance of all skin lesions.

Keywords: Acute myeloid leukaemia, lomeguatrib, O^6-Methylguanine-DNA methyltransferase (MGMT), MGMT inhibitors, Temozolomide (TMZ), triazene compounds, chemical xenogenization (CX).

INTRODUCTION

Acute myeloid leukemia (AML) is a type of acute leukemia seen predominantly in adults, particularly in elderly subjects and with a slight male prevalence. Age remains the most relevant prognostic factor for AML in that disease prognosis worsens with age, independently of other factors [1]. In spite of the use of bone marrow transplantation and high-dose chemotherapy and radiotherapy, clinical studies over the last ten years show complete response (CR) rates to chemotherapy in AML patients not exceeding 60-80% and long-term survival of no more than 25-35% [1, 2]. In addition, older adults are more likely to experience treatment-associated toxicity and less likely to benefit from treatment when undergoing standard induction and post-remission therapy [1].

In recent years, new regimens and novel agents are being studied in an effort to improve overall survival [1, 2], including cell-cycle regulating agents [3]. However, the early emergence of malignant cell resistance to antitumor agents and the absence of effective host immune responses against leukemic cells impact negatively on the therapeutic outcome presently obtainable in this haematological disorder.

A unique aspect of disease manifestation in AML patients is the so-called "Leukemia Cutis" [4, 5] in which the skin is heavily infiltrated by blast cells in various body areas. Previous investigations show that this type of lesion is particularly resistant to drug therapy. One possibility for this failure to control skin localization of blast cells could be the pharmacokinetic properties of antineoplastic agents used, which might not be able to reach adequate cutaneous concentrations.

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1. TRIAZENE COMPOUNDS

Triazene compounds (TZC) of clinical interest [i.e. Dacarbazine and Temozolomide (TMZ) (Fig. **1**)] are alkylating agents that show antitumor activity against a variety of experimental neoplasias [6], melanoma [7], brain tumours [8] and acute leukemia [9-17]. Their mechanism of action is different from that of antimetabolites, anthracyclines and other antineoplastic agents currently used in the treatment of acute leukemia. Therefore, they appear to be potentially good candidates for the management of refractory or relapsing leukemia that is resistant to standard chemotherapy.

1.1. Dacarbazine

Dacarbazine [5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; (Fig. **1**)] was synthesized as a result of a rational attempt to develop a drug that was able to interfere with purine synthesis. Dacarbazine is a prodrug that requires metabolic activation [18], principally in the liver, by cytochrome P450 (CYP450) isoforms, including CYP1A1, CYP1A2, and CYP2E1 [19]. The activation process consists in the formation of a hydroxymethylated compound, 5,(3-hydroxymethyl-3-methyl-1-triazeno)imidazole-4-carboxa mide (HMTIC). Loss of formaldehyde converts this to the monomethyl derivative, methyl-triazeno-imidazolecarboxa mide (MTIC). Spontaneous cleavage of MTIC yields the 5-aminoimidazole-4-carboxamide (AIC), and an alkylating moiety, diazomethane, which spontaneously produces molecular nitrogen and methyldiazonium cation [6, 19] (Fig. **1**). This cation is highly reactive and binds nucleophilic centers of DNA bases forming methyl adducts. In quantitative terms, the most frequent site of alkylation is the N^7- position of guanine [20] However, the minor product (~9%) of the total reaction products), O^6-methylguanine (O^6-meG) is currently considered to be the main lesion responsible for the cytotoxic and mutagenic effect of TZC [6, 20-22] as illustrated in Section 2.

1.2. TMZ

In contrast to Dacarbazine, TMZ spontaneously hydrolyses in aqueous solution to MTIC (Fig. **1**) and is active *in vitro* [22]. Moreover, the drug can be successfully administered by both parenteral and oral routes, and has been utilized in a number of clinical trials [23, 24]. Since TMZ does not require metabolic activation by the liver, it is also active in the case of liver function impairment and has wide potential clinical applications including its use for loco-regional therapy. Most importantly, TMZ but not Dacarbazine or MTIC, crosses the blood-brain barrier. Therefore, it can be used extensively in the treatment of malignant lesions in the brain. Today, because of these and other characteristics, TMZ is almost entirely replacing Dacarbazine in the clinic [25-27].

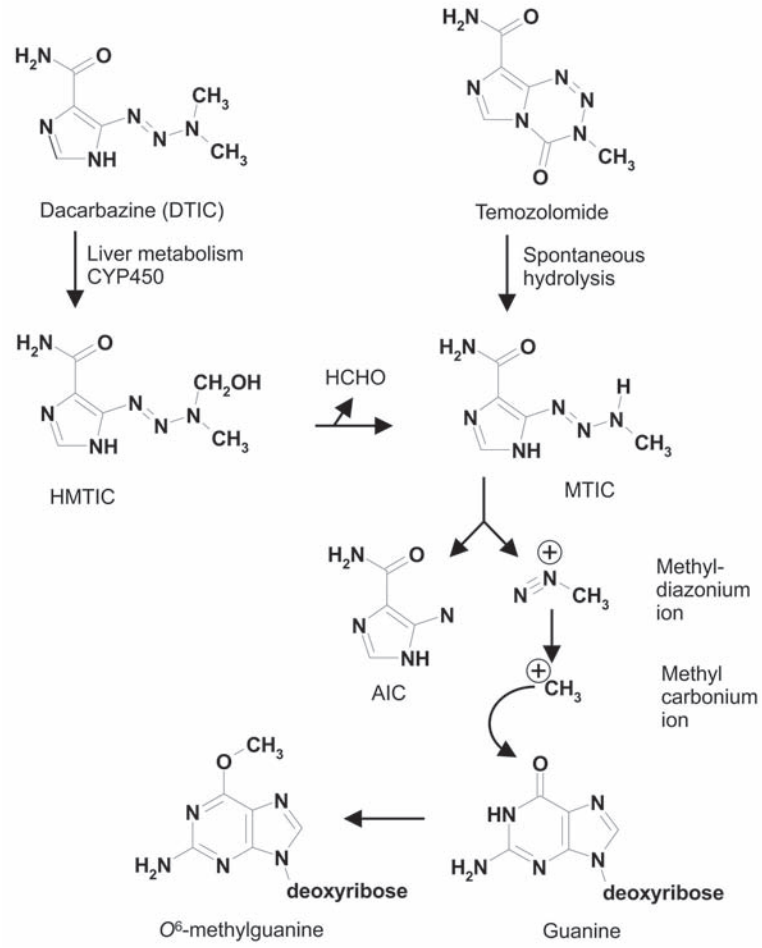

Fig. (1). Mechanism of action of Dacarbazine and TMZ. HMTIC (5,3-hydroxymethyl-3-methyl-1-triazeno)imidazole-4-carboxamide; MTIC (methyl-triazeno-imidazolecarboxamide); AIC (5-aminoimidazole-4-carboxamide).

2. CHEMICAL XENOGENIZATION (CX)

In the early seventies, a special feature of the pharmacodynamic activity of TZC was discovered by our group [28-32]. We demonstrated the appearance of novel strong transplantation antigens in various types of murine leukemia following *in vivo* treatment of tumor-bearing mice with TZC [28-30] or *in vitro* exposure of malignant cells to metabolically-activated Dacarbazine [31]. This phenomenon, which has been called "chemical xenogenization" (CX [33]), is the result of drug-induced somatic mutations [32, 34] leading to the appearance of altered proteins. In syngeneic hosts, endocellular processing of these proteins generates non-self peptides that are presented by tumor cells in association with the "Class I" major histocompatibility complex (MHC). Non-self peptides elicit MHC-restricted cytotoxic T cells [35] that are able to lyse target malignant cells *in vitro* [30, 35-37]. In addition, the presence of CX-dependent antigens in human malignancies has been suggested by investigations using human lung cancer cells *in vitro* [38], and amplification of T-cell repertoire diversity has been observed in patients vaccinated against melan-A peptide and treated with Dacarbazine [39]. Of particular note are the results of *in vivo* studies in mice showing that cytotoxic lymphocytes directed against TZC-treated leukemic cells are able to suppress the same targets in host organs, including those infiltrating the brain [40, 41].

The biochemical mechanisms underlying the antitumor and CX-inducing effects of TZC illustrated in (Fig. **2**), can be attributed to the formation of O^6-MeG in DNA [42]. This is supported by the observation that the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT), which removes the methyl group from O^6-MeG, attenuates the cytotoxic [42, 43] and CX-inducing [44] effects of TZC (Fig. **2**). During DNA replication, the presence of unrepaired O^6-MeG can result in the formation of O^6-MeG:T mispairs. These can be recognized by the post replication mismatch repair (MMR) system [45, 46], and two possible consequences have been described. According to the "futile repair" model, processing of O^6-MeG:T mispairs by the MMR system activates a signalling cascade resulting in cell cycle arrest at the G_2 phase of the second DNA replication, which is followed by either apoptosis mitotic catastrophe or a senescence-like state [47-49]. According to the "signalling" model, the MMR system transmits the damage signal directly to the checkpoint machinery, without the need for further DNA replication. Cells with a defective MMR system are highly resistant to TZC and other O^6-G-methylating agents regardless of their MGMT activity. However, in MMR-deficient cells, O^6-MeG:T mispairs can undergo further DNA replication to generate GC→ AT transition mutations, and these could be responsible for the CX phenomenon [34].

3. MGMT INHIBITORS

A large number of O^6-guanine derivatives and related compounds that can inactivate MGMT have been described [50-53]. With all agents that have been tested, inactivation takes place by transfer of the O^6-alkyl group from the inhibitor to Cys 145 in the active centre of the protein, accompanied by the release of a stoichiometric amount of guanine as illustrated in (Fig. **3**) [52]. This process, like the transfer of methyl groups from O^6-MeG in DNA, is irreversible, so until more MGMT is synthesized *de novo*, cells treated with these agents are devoid of active MGMT, and are thus susceptible to the genotoxic effects of TZC. There is some evidence that, after alkyl group transfer, MGMT undergoes ubiquitination and proteasome-mediated degradation [54].

Two MGMT inactivating agents, O^6-benzylguanine (O^6-BG) and more recently O^6-(4-bromothenyl)guanine (Lomeguatrib, LM) (Fig. **4**), have been used in various clinical trials in combination with a number of O^6-alkylating agents and Irinotecan [52]. In preclinical [55-58] and clinical [14, 59-63] studies, LM was found to be orally bioavailable, highly active and minimally toxic. Moreover, taking into account that TMZ is largely used in malignant diseases in the brain, of particular interest is the finding that LM is able to cross the blood-brain barrier.

4. CLINICAL STUDIES ON THE ANTILEUKEMIC ACTIVITY OF TRIAZENES

A limited number of clinical trials concerning the use of TZC in acute leukemia is presently available in the literature [9-17] (Table **1**). In general, a reasonable number of responses in terms of blastolysis, or effective clinical responses has been described, although up to now no large-scale randomized clinical studies have been conducted to established whether TZC treatment provides definite survival improvement over conventional therapy. It must be pointed out that the probability of successful response to TMZ is higher in AML than in other forms of leukemia since limited MGMT activity is present in leukemic blasts in approximately 50% of AML cases. However, the strategy of patient selection based on blast-associated MGMT deficiency, or of drug-induced MGMT down-regulation, opens the way to treat successfully an appreciable number of non-AML cases as well.

Here we report a brief analysis of the therapeutic results obtained with treatment of leukemic patients with Dacarbazine or TMZ. Whenever possible, we will consider also the role played by MGMT in host's response to TZC administration.

A pilot clinical study on the antileukemic activity of TZC was performed by our group for the first time in 1992 [9], when 9 patients affected by relapsing or refractory AML were treated with high doses of Dacarbazine. The results demonstrated that in 3 patients who were refractory to standard chemotherapy, Dacarbazine was able to induce complete blast disappearance within 7 days of the start of treatment. In this study, the leukemic blasts of the responding patients showed minimal or no MGMT activity. In order to confirm the biochemical bases of this finding, further *in vitro* studies were conducted by our group on leukemic cells obtained from 60 patients with acute leukemia, 48 bearing AML, 5 bearing chronic myelogenous leukemia in blast crisis and 7 with acute lymphoblastic leukemia (ALL) [64]. The results of this investigation showed a highly significant inverse correlation between blast susceptibility to the suppressive effects of TMZ and MGMT activity levels. On the other hand, no relationship was found between chemosensitivity of blast cells to Ara-C and expression of MGMT.

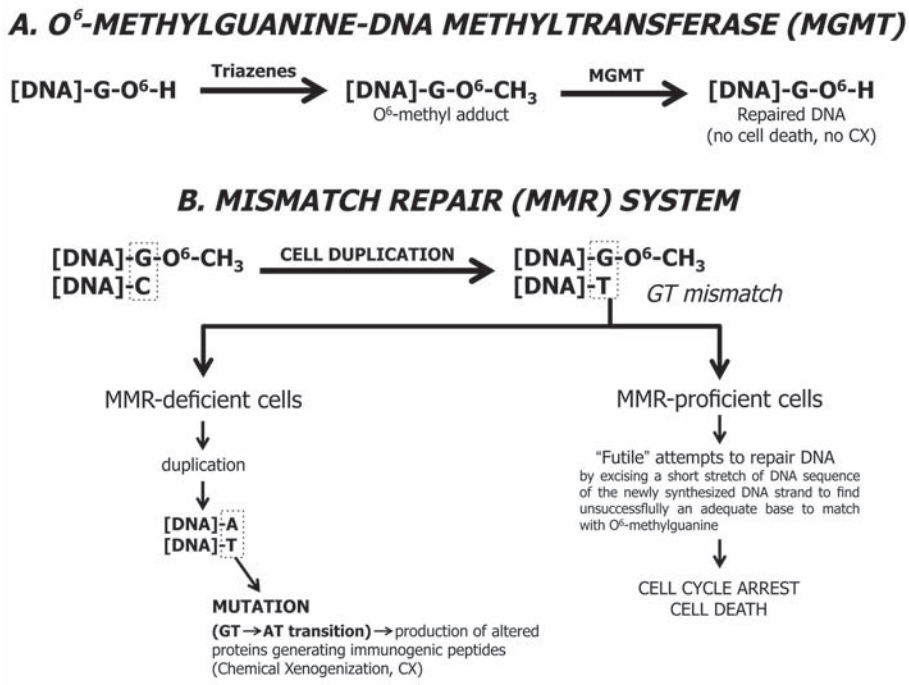

Fig. (2). Mechanisms of "Chemical Xenogenization" (CX) and cell cycle arrest/cell death induced by triazene compounds in proliferating cells.

The pharmacokinetics properties of TMZ, render this agent more attractive than Dacarbazine for the treatment of acute leukemia. For this reason, *Seiter et al.* [10] explored the use of TMZ in AML in 2002. In this pilot study conducted on 20 patients, the Authors obtained 10% complete response (CR) and additional 10 CR without complete platelet recovery and almost 50% of the patients showed a significant decrease in the abundance of bone marrow blasts after TMZ treatment. Moreover, the drug was well tolerated and hence demonstrated a significant antileukemic activity along with a substantial therapeutic index when administered as a single agent. However, additional studies by the same group on 14 patients with myelodysplastic syndrome (MDS) [11], did not show therapeutic benefit. No data on MGMT levels and the functional status of the MMR system in blast cells from these patients were reported by the Authors, so we cannot rule out the possibility that the lack of response to TMZ in this limited cohort of patients might be ascribed to blast-associated high MGMT activity and/or MMR deficiency.

A subsequent study by *Horton et al.* [12] in 2007, reported the results of a pharmacokinetic and pharmacodynamic investigation of TMZ in pediatric patients with refractory or recurrent leukemia. Twenty-four patients, 16 with ALL and 8 with AML were treated with 200 or 260 mg/sqm TMZ orally for 5 consecutive days. The drug was well tolerated even at the highest dose employed. Two patients responded well to treatment without, however, attaining CR but partial response only. In both, blast cells were MGMT deficient and the MGMT promoter was methylated; they were, presumably, MMR-proficient. It is well known that MGMT activity in leukemic blasts displays high individual variability, although the mean MGMT activity of ALL blasts is significantly higher than that of AML blasts. Since pediatric patients are predominantly affected by ALL, TMZ response rate would be expected to be low in this group, which might therefore be anticipated to benefit from the appropriate use of MGMT inhibitors.

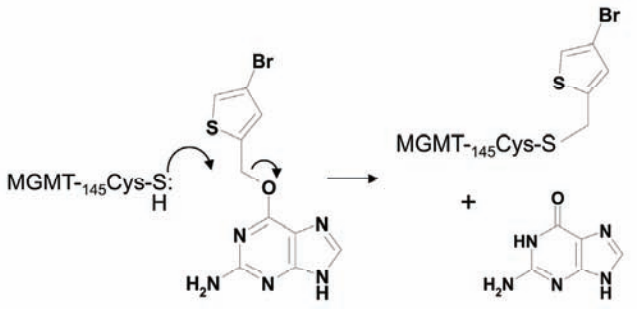

Fig. (3). Mechanism of action of O^6-(4-bromothenyl)guanine (Lomeguatrib, LM). LM acts as a pseudosubstrate inactivator of MGMT. The bromothenyl group is irreversibly transferred to the cysteine (Cys 145) thiol group of MGMT to form the *S*-bromothenyl derivative which can no longer react with an O^6-alkylated guanine residue in DNA, leaving such lesions unrepaired.

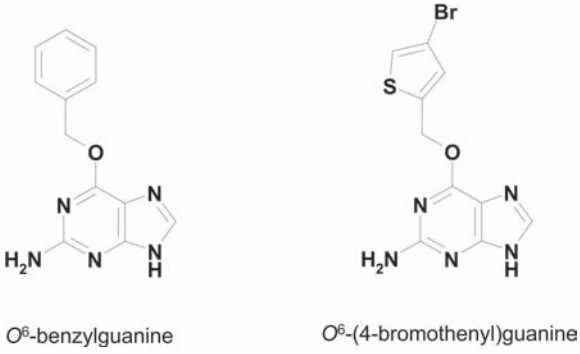

Fig. (4). Chemical structure of O^6-benzylguanine (O^6-BG) and O^6-(4-bromothenyl)guanine (Lomeguatrib, LM).

Table 1. Clinical Studies with Triazene Compounds in Patients with Leukemia

Study [Ref]	Disease (No. pts)	Median Age (Range)	Treatment Description	Type of Response	Response Rate %
Franchi *et al.* 1992 [9]	Relapsed/refractory AML (9)	43(22-60)	DTIC 400-800 mg/sqm i.v. (day 0, 1, 2)	Complete blastolysis in PB and BM	33
Seiter *et al.* 2002 [10]	Relapsed/refractory AML (16), ALL (2) CML-BP(2)	44 (21-79)	TMZ 200 mg/sqm p.o. (day 1-7 or day 1-9)	CR CR (except for platelet recovery) Partial blastolysis in BM	10 10 45
Seiter *et al.* 2004 [11]	MDS (14)	71 (48-79)	TMZ 200 mg/sqm p.o. (day 1-7, repeated every 5-6 weeks)	CR PD NC in BM	0 28 57
Horton *et al.* 2007 [12]	Relapsed/refractory ALL (16), AML(8)	11 (1-19)	TMZ 200 mg/sqm p.o. or 260 mg/sqm p.o. (day 1-5)	PR PD	12 88
Brandwein *et al.* 2007 [13]	AML (46)	73 (60-83)	TMZ 200 mg/sqm p.o. (day 1-7).	CR CRp PR (All CR and PR were obtained in patients bearing blast with negative or low MGMT levels)	7 4 4
Caporaso *et al.* 2007 [14]	Refractory AML (8)	55 (38-70)	LM 40 mg/sqm p.o. (day 1-10); TMZ 150 mg/sqm p.o. (day 1-7), followed by IL-2, 1 MIU/s.c. (day 15-25)	Partial or complete blastolysis in PB or in BM (All LM treated patients showed a marked decrease of MGMT activity in peripheral blasts)	75
Seiter *et al.* 2009 [15]	Relapsed/refractory AML (15), ALL (3) Biphenotypic (2)	52 (24-73)	CDDP 50 to 100 mg/sqm i.v. (day 1) plus TMZ 200 mg/sqm p.o. (day 1-7)	CR Partial blastolysis in BM	5 10
Rizzieri *et al.* 2010 [16]	Relapsed/refractory AML (33), ALL(2)	61 (22-80)	TMZ 300 mg p.o. (day1-5) laromustine 300 mg/sqm i.v. (single injection)	CR Morphologic, leukemia-free, but persistent hypocellular BM status.	8 11
Medeiros *et al.* 2012 [17]	Untreated high-risk/relapsed/refractory AML (36)	75 (64-87)	TMZ 100 mg/sqm p.o (day 1-14) followed by TMZ 200 mg/sqm p.o. (day1-7)	CR CRp LFS Induction death Resistant disease	22 8 6 25 47

ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; BM, bone marrow; CDDP, cisplatin; CML-BP, chronic myelogenous leukemia in blastic phase; CR, complete remission; CRp, complete remission with incomplete platelet count recovery; DTIC, Dacarbazine; IL-2, interleukin-2; i.v., intravenously; LFS, leukemia free state; LM, Lomeguatrib; MDS, myelodysplastic syndrome; MGMT, O^6-methylguanine-DNA methyltransferase; MIU, Million International Units; NC, no change; PB, peripheral blood; PD, progressive disease p.o., per os; PR, partial response; pts, patients; Ref, references; s.c., subcutaneously; TMZ, Temozolomide. References are given in brackets.

In 2007, *Brandwein et al.* [13] published the results of a Phase II study of TMZ in 46 poor prognosis AML patients aged 60 years or older. In most cases, these patients do not tolerate highly aggressive therapy, especially when they suffer from adverse co-morbid medical conditions. It must be stressed that supportive care alone is associated with a median survival generally not exceeding 4 months. Due to its limited non-hematological toxicity, TMZ was considered a reasonable chemotherapeutic option being less aggressive than standard induction therapy with cytarabine in combination with an anthracycline compound. Considering the essential role played by MGMT in TMZ resistance, the authors determined the MGMT protein level of the blasts of their patients by western blot analysis and a biochemical classification was reported, based on the MGMT/Actin ratio determined by densitometric analysis of band intensity. In this classification, MGMT levels were considered "negative", "weak" or "strong" at MGMT/Actin ratio of less than 0.2, from 0.2 to 0.4, or > 0.4 respectively. Induction therapy consisted of TMZ 200 mg/sqm p.o. daily for 7 days. Patients achieving CR or CR with inadequate platelet recovery (CRp) received up to 6 monthly post-remission cycles of TMZ (200 mg/sqm p.o. daily for 5 days). In this study, the overall CR rate was 11%, and the overall response rate was 15%. All CR and 2 of the 3 partial remissions (PR) were obtained in patients in which leukemic blasts were "negative" for MGMT, whereas 1 PR only was seen in a patient showing "weak" blast MGMT levels. In contrast, no patient with "strong" MGMT-positive blasts responded to TMZ. At the ASH Annual Meeting, these authors presented a poster [65] showing

the frequency of patients with AML or MDS who could potentially benefit from TMZ treatment. They found that 41% of 128 leukemic patients [median age 76 years old (range 46-94 years), affected by AML (84%) or MDS (16%)] were "negative" for MGMT expression, and therefore good candidates for successful treatment with TMZ.

Preclinical and clinical investigations have demonstrated that a number of inhibitors can consistently reduce the levels of MGMT activity in cells and tissues. This has opened up a new strategy to successfully treat human leukemia cells that are triazene-resistant by virtue of expressing high MGMT levels. This currently involves the sequential treatment with an MGMT inhibitor, or more correctly, inactivator, followed by TZC administration [52]. However, prior to this, other strategies were examined. Thus in 2009, on the basis of preclinical studies performed in our laboratory showing that Cisplatin was able to down-regulate MGMT expression in malignant cells [66, 67], *Seiter et al.* [15] treated 20 leukemic patients (15 AML, 3 ALL, 2 biphenotypic leukemia) with Cisplatin (escalating dose from 50 to 100 mg/sqm day 1) together with TMZ (200 mg/sqm p.o. day 1-7). Treatment was well tolerated even at the highest cisplatin dose. One patient experienced CR and two other patients showed almost complete disappearance of blast cells. However, this was not accompanied by bone marrow recovery.

In addition to this, clinical studies were conducted exploiting TMZ itself as an agent that is able to induce MGMT inactivation, as evidenced in *in vitro* models [67, 68]. In 2010 *Rizzieri et al.* [16] subjected 35 leukemic patients to TMZ (300 mg/day p.o. for 5 days) pretreatment in order to inactivate MGMT, and this was followed by Laromustine administration (300 mg/sqm as a single i.v. injection). This strategy was based on the observation that the antitumor activity of Laromustine (Cloretazine), a bifunctional alkylating agent targeting O^6-G and proposed as salvage therapy in refractory AML in elderly people [69], is entirely suppressed by MGMT. Three patients with previously refractory disease achieved CR, and 4 patients achieved a morphologic, leukemia-free, but persistent hypocellular bone marrow status.

Quite recently, *Medeiros et al.* [17] adopted the strategy to down-regulate MGMT expression by limited daily doses of TMZ (100 mg/sqm/day for 14 days) followed by induction treatment with high TMZ dose (200 mg/sqm/day for 7 days) for 6 monthly cycles. The Authors enrolled 36 high-risk AML elderly patients (median age, 75 years), and divided them into 2 cohorts according to whether the MGMT promoter was methylated (mMGMT) or unmethylated (unMGMT). This classification was taken as an indicator of patients with low MGMT activity (i.e. mMGMT) or high MGMT activity (i.e. unMGMT). The strategy of low "priming" TMZ doses followed by high doses of TMZ was applied to the majority of patients (86%) who were found to be unMGMT. The other patients bearing leukemic blasts with presumably low MGMT activity (i.e. mMGMT) were treated only with the high TMZ dose (i.e. 200 mg/sqm/day for 7 days). The overall response rate was 36% (CR 22%, CRp 8%, limited disease-free period 6%), induction death 25%, resistant disease 47%. The median overall survival was 11.5 weeks (range 1.5-110 weeks), and the mean survival was 24 weeks.

One potential problem associated with the use of TMZ itself as MGMT inhibitor, is the possibility that protracted low-dose administration would engender the emergence of highly TMZ resistant neoplastic cell clones [70]. For this reason, the use of non-triazene inhibitors of MGMT could be mandatory.

In a pilot clinical study performed by our group [14], 8 patients with refractory AML were subjected to LM treatment (40 mg/sqm/day p.o. from day 0 through day 10 in order to inactivate MGMT) and to TMZ administration (150 mg/sqm/day p.o. from day 1 through day 7), followed by immunoenhancing treatment with interleukin-2 (IL-2, 1 MIU/day subcutaneously, from day 15 through day 25). Six out of 8 patients showed partial or complete disappearance of blast cells in peripheral blood or in bone marrow. Side effects consisted in severe and long-lasting myelosuppression, accompanied by limited non-haematological toxicity. Two patients survived beyond 10 months, 4 died of opportunistic infections and 2 of progressive disease. Although the number of patients was extremely low, this study strongly suggests that blast can be made highly sensitive to TMZ if MGMT activity is suppressed by LM.

5. CASE PRESENTATION

5.1. Clinical Data

The present case report concerns the clinical history and the therapeutic approach adopted in a patient affected by AML accompanied by marked skin lesions due to massive blast infiltration. This patient did not have an HLA-compatible donor for allogeneic bone marrow transplantation. Moreover, he was not eligible for conventional chemotherapy (see below). Therefore, the patient was enrolled in a pilot clinical study performed by our group, based on an experimental approach of immuno-chemotherapy with LM associated with TMZ, followed by IL-2 administration [see ref 14, case # 4]. The cytokine was employed with the intent to protect from drug-induced immuno-depression and promote host's cell-mediated immune response against residual leukemic cells, possibly surviving and antigenically altered (i.e. subjected to CX) after drug treatment. Actually, IL-2 is one of the main cytokines able to promote proliferation of T lymphocytes and NK cells, that can be involved in resistance to infection [71] and cell-mediated responses against tumor cells [72]. In particular, previous studies performed in our laboratory showed that IL-2 was able to counteract TZC-induced immunodepression [73] and amplify the effect of Dacarbazine in a mouse lung cancer model [37].

The combined use of antineoplastic agents and cytokines in malignant diseases has been studied in a number of clinical trials. In particular, IL-2 in combination with TZC have been used in the bio-chemotherapy of melanomas [74-83]. Although this type of treatment was well tolerated and produced occasionally high remission rate, no clear advantage of immuno-chemotherapy over chemotherapy alone was found in terms of overall survival, except for the remarkable but preliminary results reported by *Fateh et al.* [81]. Moreover IL-2 has been employed with the intent to amplify host's immune reactivity against autochthonous tumor in the chemo-immunotherapy of colon cancer [84], with promising results.

The patient, (58 year-old, male) was admitted to our hospital following 8 month history of blood dyscrasia [WBC**:** 7.2 x 10^9/l (neutrophils 32%, lymphocytes 55%, monocytes 13%); Hb: 9.1 g/dl; MCV: 87 fL; RDW: 19.4%; platelets: 60 x 10^9/l], leading to the diagnosis of primary (idiopathic) osteomyelofibrosis at fibrotic stage with extensive osteosclerosis. He had no significant past medical history and denied any previous chemical or radiation exposure.

At the time of admission, the patient presented debilitating physical conditions and complained weight loss, fever and night sweats. Physical examination showed no lymphadenopathies, modest hepatomegaly and pronounced splenomegaly. Moreover, he presented multiple cutaneous lesions (mainly on face, arms and back) accompanied by thickening of facial skin and gingival hypertrophy. Peripheral blood counts confirmed anemia (Hb 8.0 g/dL), thrombocytopenia (23 x 10^9/l), and revealed a huge increase of WBC (120 x 10^9/l). Blood chemistry was within the normal limits, except for lactate dehydrogenase that was 860 Units/L (x 2 the upper normal value).

Peripheral blood smear showed the presence of 95% of myeloid blasts with cytoplasmatic granules and one or more nucleoli, resulting positive for myeloperoxidase (66%), sudan black B (66%) and alpha-naphthyl butyrate esterase (30%) and negative for periodic acid Schiff stain at cytochemical analysis. Myeloid blasts resulted positive for CD33, CD117, CD13, CD14, CD15 and CD3 antigens and negative for CD34, HLA-DR, CD19 and CD10 at flow cytometry analysis.

Bone marrow aspirate did not yield adequate amount of bone marrow cells, while bone marrow biopsy showed a massive infiltration of blast cells expressing myelomonocytic pattern at immunohistochemistry within the context of frankly sclerotic marrow. Moreover, the presence of dysplastic megacariocytes and of a reduced number of erythroblasts was noticed. Molecular characterization of malignant cells indicated that they were negative for RUNX1-RUNX1T1, CBFβ/MYH11, BCR-ABL, DEK-NUP214 and PTD-MLL, while cytogenetic analysis of blasts failed.

All together, these findings indicated that the patient had developed an acute myelo-monocytic leukemia, M4 (according to FAB classification), resulting from blastic transformation of previous primary myelofibrosis. Cutaneous biopsy confirmed the massive infiltration of skin by myeloperoxidase-positive and CD34-negative blast cells, as illustrated in (Fig. **5**). Total-body CT scan confirmed the presence of splenomegaly and liver enlargement.

When diagnosis was completed, no suitable bone marrow donor was available to adopt highly efficient treatment protocols [85, 86] and the patient underwent cytoreductive chemotherapy consisting in hydroxyurea (2g/day) along with 6-mercaptopurine (50 mg/day) for 27 days. In spite of dose escalation of both drugs, no satisfactory control of leukocytosis was attained, and no change of the serious skin lesions was observed. The poor performance status at the time of admission (ECOG PS 3) indicated that the patient was not able to tolerate the non-hematological toxic effects of intensive conventional induction chemotherapy. Moreover, it was considered that conventional chemotherapy is followed by low rate of overall responses and poor clinical outcome in patients with blastic transformation of primary myelofibrosis at fibrotic stage with extensive osteosclerosis. Therefore, a novel experimental protocol [14], approved by our Ethic Committee, was proposed to the patient who gave written consent to treatment. The patient received LM (40 mg/sqm/day p.o. from day 0 to day 10), TMZ (150 mg/sqm/day p.o. from day 1 to day 7), followed by IL-2 administration (1 MIU/day subcutaneously, divided into 2 doses injected in 2 different sites each day) from day 15 to day 25.

The clinical outcome, relative to the first cycle of treatment, showed that no myeloid blasts were detectable in the peripheral blood of the patient on day 8 at the end of TMZ administration. Remarkably, patient general conditions improved dramatically, and all skin lesions, including the disfigured face, disappeared. As a consequence, it was not possible to identify a suitable region for a comparative biopsy and no further skin biopsy was considered to be appropriate. At the end of IL-2 administration, a bone marrow aspirate showed the presence of only 1% of blasts, whereas leukaemic cells were not detectable in the peripheral blood. Non-haematological side effects were minimal (headache, grade 1 constipation, and grade 1 hepatotoxicity). However, no complete haematological recovery was noticed after the start of therapy. Since the patient presented a persistent severe neutropenia, he was not eligible for a second cycle of drug treatment. On day 57 the patient experienced an early leukemia relapse (12% blasts in the peripheral blood). On day 70 peripheral blood cell count showed Hb 6.6 g/dL, platelets 6 x 10^9/l, WBC 3.05 x 10^9/l with over 50% of circulating blasts. However, no skin lesions were detectable. On day 73 the patient died for pneumonia, confirmed by CT scan of the lungs, caused by combined *Aspergillus fumigatus* and *Pseudomonas Aeruginosa* infection documented by bronchoalveolar lavage cultures.

5.2. Biological Studies

Patient' blasts were collected before LM treatment and 24 h after the first administration of the MGMT inhibitor, immediately before starting TMZ treatment, and stored in liquid nitrogen for the subsequent Western blot analyses. Leukemic blasts were tested for the presence not only of MGMT protein, but also for the proteins of the MMR complex that plays a crucial role in malignant cell sensitivity to TMZ [45, 46, 48, 49, 70]. Leukemic blasts collected before LM administration were examined for MMR protein expression (Fig. **6A**). Extracts from the MMR-deficient TMZ-resistant colon cancer cell lines Lovo and HCT116, and the MMR-proficient TMZ-sensitive lymphoblastoid cell line TK6 were used as controls. Lovo cells do not express the hMSH2 protein because they are homozygous for a partially deleted *hMSH2* gene, whereas HCT116 cells do not express the hMLH1 protein because they harbor a hemizygous nonsense mutation in the *hMLH1* gene [87]. The hMSH6 protein is undetectable in Lovo cells since it is unstable in the absence of hMSH2. Moreover, hPMS2 protein is expressed at very low levels in HCT116 cells because it is unstable in the absence of hMLH1. Instead, all MMR proteins were present in the patient's blasts, although hMSH3 and hMSH6 were expressed at a low level. This finding confirms our previous preliminary data [14] and is consistent with patient clinical

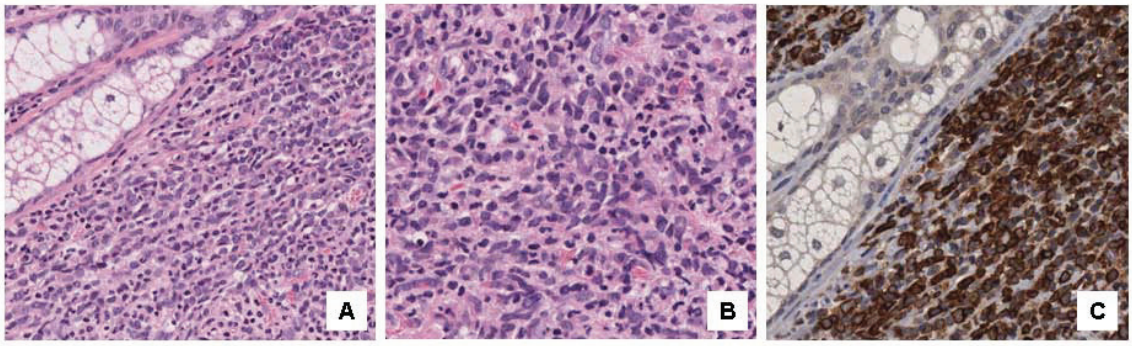

Fig. (5). Histological analysis of patient's skin biopsy. **A**) Dermal heavy periappendageal infiltration by medium-sized atypical round to ovoidal leukemic myeloid blasts (Hematoxylin & eosin stain, 200X). **B**) Dermal infiltration by medium-sized atypical round to ovoidal leukemic myeloid blasts (Hematoxylin & eosin stain, 400X). **C**) Strong cytoplasmic (brown) stain in neoplastic cells for Myeloperoxidase (Immunohistochemistry, 200X).

response to therapy. Leukaemic blasts collected before and after LM administration were comparatively analyzed for protein expression and activity of MGMT. A substantial reduction of both protein level (Fig. **6B**) and activity (see legend of Fig. **6B**) of MGMT was detected in peripheral blood blasts collected 24 h after LM administration. Additional studies were performed to evaluate the level of *MGMT* gene transcript by quantitative Real-Time RT-PCR, before and after LM administration. The results (Fig. **6C**) show that a limited amount of *MGMT* mRNA was detectable in patient's blasts before LM treatment, in accordance with the scarce quantity of MGMT protein detected in Western Blot analysis. Unexpectedly, 24h after LM administration, significant reduction of *MGMT* transcript was also detected in patient's blasts (Fig. **6C**).

To investigate the kinetics of MGMT depletion and recovery after LM treatment, the MGMT-proficient human T leukemia Jurkat cells were cultured in the presence of 10 μM LM for 24 h and analyzed for MGMT protein expression after 1, 3, 6 and 24 h of drug exposure. At the end of the incubation period, the cells were recovered, washed and suspended in fresh culture medium. The MGMT protein levels were then evaluated after 3, 6 and 9 days of culture. Upon LM exposure, a manifest decline of MGMT protein expression occurred as early as 3 h from the start of LM treatment, followed by complete disappearance of the protein after 24 h of culture in the presence of the MGMT inhibitor (Fig. **6D**). After LM removal, MGMT protein expression re-emerged on day 3 of culture and increased after 6, and 9 days without, however, reaching control levels (Fig. **6E**) These results were confirmed in 2 additional independent experiments. Further studies were performed in order to explore the pattern of *MGMT* gene transcription in this *in vitro* model comparatively with that observed in patient's blasts using Real-Time RT-PCR. The results (Fig. **6C**) show that significant decline of MGMT transcript after 24 h exposure to LM was also detectable in Jurkat cells.

DISCUSSION

The present AML case showing an additional "Leukemia Cutis" pattern, allows different considerations that could be of guidance for future application of TZC in the therapy of haematopoietic malignant diseases. First of all, in accordance with preclinical and clinical evidence furnished by the literature [14, 52, 58, 62], administration of LM was able to essentially abolish MGMT activity and protein expression, with a mechanism similar to that involved in the suppressive activity of another MGMT inhibitor of clinical interest, namely O^6-BG [50]. Reduction of MGMT protein levels after LM administration, could be ascribed to rapid degradation of the enzyme by a proteasome-dependent pathway that is activated by alkylated enzyme, as previously described [54].

From the clinical point of view, it must be pointed out that rapid and profound blastolysis occurred following LM plus TMZ administration. However, the treatment was well tolerated, probably much better than that observed with other myeloablative procedures (e.g. busulfan), and was not accompanied by notable non-hematological toxicity. This result means that the target leukaemic cells did not only express low or absent MGMT activity after treatment with LM, but also displayed a fully functional MMR system. Of great interest was the observation that the multiple and deep skin lesions infiltrated by myeloblasts responded quickly to therapy and no skin relapse was observed in concomitance with aggressive and rapid re-growth of leukaemic cells in bone marrow and peripheral blood at 2 months after the onset of chemo-immunotherapy. It is reasonable to suggest that TMZ was particularly active against malignant cells in the skin for pharmacokinetic reasons, i.e. for a possibly elevated cutaneous tropism of the agent. This hypothesis could be supported by the numerous data of the literature showing that TMZ has been used with appreciable results in the treatment of different types of lymphomas, including not only primary CNS lymphoma [88], but also cutaneous lymphoma [89-91]. However, we cannot exclude a possible role of antigen-dependent [92] or natural immunity [93] present in the skin and directed against resident TMZ-treated leukaemic cells. If this was the case, it is possible to draw an immune-chemotherapy scenario in which residual disease surviving the first cycle of LM+TMZ+IL-2 treatment was not adequately controlled in the haematopoietic organs by supposedly weak systemic immunity. On the contrary, it is reasonable to hypothesize that a stronger skin-associated resident immune response could have controlled more efficiently residual blast cells, possibly antigenically modified by local exposure to MTIC, the active metabolite of TMZ [6].

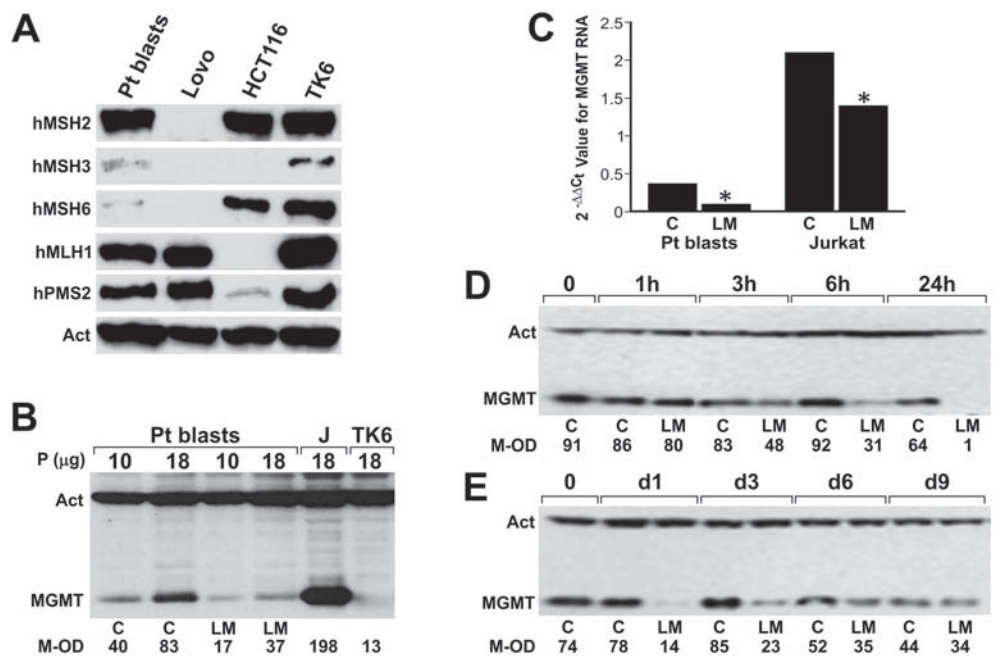

Fig. (6). Western blot and Real Time RT-PCR analysis of patient's blasts and of *in vitro* growing malignant cells. Patient's blasts, obtained from the peripheral blood and separated by centrifugation on Ficoll Hypaque gradient, and tumour cell lines, were lysed and protein or RNA extracted as previously described [97, 98]. The Figure illustrates the results of a representative experiment confirmed by at least 3 independent experiments that furnished comparable data. **A**) **MMR protein expression in patient's blasts (Pt blasts) collected before the first cycle of LM administration.** Extracts from the colon cancer cell line Lovo, which does not expresses hMSH2, hMSH3 and hMSH6 , the colon cancer cell line HCT116, which does not express hMSH3 and hMLH1 and expresses low levels of hPMS2, and the MMR-proficient lymphoblastoid cell line TK6 were used as controls. Reagents, antibodies and materials utilized have been previously described [99]. **B**) **MGMT expression in patient's blasts collected before and 24 h after LM administration (40 mg/sqm).** The MGMT-proficient cell line Jurkat and the MGMT-deficient cell line TK6 were used as a positive and negative control, respectively. In this case, and in the experiments reported in panel D and E, protein separation was performed by running cell extracts on pre-cast gels (NuPAGE^{R} Novex Bis-Tris, Invitrogen, Life Technologies, Grand Island, NY) with 10% polyacrylamide, using XCell SureLock^{TM} Mini-Cell apparatus (Invitrogen) following the instructions of the producer. At the end of the electrophoretic separation, proteins were transferred to nitrocellulose filters by electrotransfer (miniblot apparaturs, Invitrogen). The membrane were then incubated with an anti-actin rabbit polyclonal antibody (Sigma St Louis, MO, USA) and mouse monoclonal antibody against MGMT (Millipore, Billerica, MA). Bands were developed using westernBreeze chemiluminescent immunodetection system (Invitrogen), according to the manufacturer's instructions. Immunoblot was scanned by densitometer and the optical density of MGMT band (M-OD) was expressed as arbitrary units. P, μg of protein loaded onto the gel. The enzymatic activity of MGMT was also evaluated in patient's blasts or in Jurkat cells, before or after 24h treatment with LM *in vivo* or *in vitro* respectively. MGMT activity was determined by measuring the transfer of ^{3}H-methyl groups from a DNA substrate to the MGMT protein, as previously described in detail [100] and modified by *Pepponi et al.* [99]. The results indicate that the enzyme activity [mean ± standard error (n. obs. 3)] of patient's blasts collected before LM administration and of Jurkat cells before incubation with LM was 107.5 ± 13.9 fmoles/mg protein and 812 ± 83.8 fmoles/mg protein, respectively. Twenty-four hours after patient treatment with LM (40 mg/sqm) or *in vitro* exposure of Jurkat cells to LM (10 μM), enzyme activity dropped to almost undetectable level in both cases. **C) Relative MGMT mRNA values of patient's (Pt) blasts, collected before (C) and after (LM) 24 h of LM administration, and of control (C) or treated (LM) Jurkat cells incubated with LM for 24h.** Total RNA was extracted, from cells using TRI Reagent solution (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. To remove contaminating DNA from RNA preparations, a turbo DNA-free KIT from Applied Biosystems was used. cDNA was synthesized using 2 μg of RNA and TaqMan RT kit (Applied Biosystems), according to the manufacturer's instructions. Real-time-PCR was performed in triplicate, as described in reference [98]. The primers and probes used for amplification and quantitation of MGMT and of the endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems. (Assay-On-Demand: catalogue n. Hs.00172470_m1 for MGMT. Catalogue n. 4326317E for GAPDH). The relative quantification of MGMT was performed using the comparative threshold cycle (C_T) method (as described by "Applied Biosystems") that uses an arithmetic formula ($2^{-\Delta\Delta CT}$). $\Delta\Delta C_T$ is the difference between ΔC_T of the sample under investigation and the ΔC_T of the calibrator sample. The RNA obtained from Jurkat cells was chosen as calibrator sample. *P<0,05 with respect to the corresponding control, according to two-tail paired "t" test analysis (number of observations relative to patient = 7, and relative to Jurkat = 11). **D) Time-course analysis of LM-induced MGMT decline in Jurkat cells**. The cells were cultured in the absence or in the presence of 10 μM LM and analyzed for MGMT expression at the indicated intervals of time. **E) Time-course analysis of MGMT recovery in LM-treated Jurkat cells after removing the inhibitor**. The cells were cultured in the absence or in the presence of 10 μM LM for 24 h. Thereafter, the cell were washed, suspended in fresh medium and maintained in culture. MGMT expression was evaluated at the end of LM treatment (d1, day 1) and after 3, 6 and 9 days of culture in the absence of the inhibitor. Act, actin; MGMT, O^{6}-alkyl-guanine-alkyl-transferase; M-OD, optical densities of MGMT band.

The failure of bone marrow recovery was probably facilitated by the myelofibrotic status of patient's bone marrow, already existing before the onset of leukemia. It is reasonable to hypothesize that the availability of suitable donor for allogeneic bone marrow transplantation [94] could have provided the basis for successful bone marrow recovery and generation of protective immunity against opportunistic infections that were responsible for patient's death.

The *in vitro* studies on human Jurkat leukemia cells pointed out that, similarly to the results obtained with patient's blasts, LM treatment produced a marked reduction of MGMT protein within 24 h. In these experiments, decline of MGMT was noticed as early as 3 h after exposure to LM. Moreover, MGMT enzymatic activity was virtually suppressed in Jurkat cells within 1 h (data not shown) in agreement with previous observations [58], leading to a rapid degradation of the enzyme by the proteasome-dependent pathway [54]. More difficult to interpret is the finding that the expression of *MGMT* gene transcript was significantly reduced either in patient's blasts and in Jurkat cells 24 h after treatment with LM. Although similar findings have been reported by *Konduri et al.* [95] in cancer cells exposed to O^6-BG, no data are presently available to elucidate the mechanism underlying this event. After LM was removed from *in vitro* cultured Jurkat cells, partial but not complete recovery of MGMT protein was detectable up to day 9 of culture, although most of the original Jurkat cell population exposed to LM was replaced by daughter cells as indicated by *in vitro* cell duplication rate (data not shown). On the other hand, further experiments not described in the present report, were performed with Jurkat cells treated with LM for 24 h, washed and exposed to X rays to impair malignant cell proliferation. In this case, non-proliferating damaged cells showed a consistent recovery of MGMT protein expression on day 3 after irradiation. Therefore, cell duplication does not necessarily imply that MGMT is rapidly re-synthesized, whereas ionizing radiations induce MGMT up-regulation even in severely damaged malignant cells, in line with previous data of the literature [96]. Should *in vitro* data be representative of *in vivo* situation, one would expect that setting of a suitable treatment protocol of LM plus TMZ necessitates great investigational efforts. Actually, no data are available on MGMT recovery kinetics in leukemic blasts after *in vivo* treatment with a single administration of LM. Similarly, the kinetics of MGMT down-regulation followed by recovery of bone marrow stem cells is presently not known. This precludes the possibility to design an appropriate treatment schedule able to exploit an hypothetical temporary "therapeutic window" in which the enzyme level is limited in malignant cells and high in normal haematopoietic cells. Therefore, further studies are mandatory for identifying a rational design of LM plus TMZ administration, quite different from a plain drug combination. In any case, the present report highlights the potential therapeutic role of TMZ not only in acute leukemia, as already demonstrated by previous investigations, but also in a case of severe skin lesions provoked by leukemic cell infiltration.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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