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Erythroid induction of K562 cells treated with mithramycin is associated with inhibition of raptor gene transcription and mammalian target of rapamycin complex 1 (mTORC1) functions

Alessia Finotti, Nicoletta Bianchi, Enrica Fabbri, Monica Borgatti, Giulia Breveglieri, Jessica Gasparello, Roberto Gambari*

Department of Life Sciences and Biotechnology, Section of Biochemistry and Molecular Biology, University of Ferrara, Italy

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ABSTRACT

Rapamycin, an inhibitor of mTOR activity, is a potent inducer of erythroid differentiation and fetal hemoglobin production in β-thalassemic patients. Mithramycin (MTH) was studied to see if this inducer of K562 differentiation also operates through inhibition of mTOR. We can conclude from the study that the mTOR pathway is among the major transcript classes affected by mithramycin-treatment in K562 cells and a sharp decrease of raptor protein production and p70S6 kinase is detectable in mithramycin treated K562 cells. The promoter sequence of the raptor gene contains several Sp1 binding sites which may explain its mechanism of action. We hypothesize that the G + C-selective DNA-binding drug mithramycin is able to interact with these sequences and to inhibit the binding of Sp1 to the raptor promoter due to the following results: (a) MTH strongly inhibits the interactions between Sp1 and Sp1-binding sites of the raptor promoter (studied by electrophoretic mobility shift assays, EMSA); (b) MTH strongly reduces the recruitment of Sp1 transcription factor to the raptor promoter in intact K562 cells (studied by chromatin immunoprecipitation experiments, ChIP); (c) Sp1 decoy oligonucleotides are able to specifically inhibit raptor mRNA accumulation in K562 cells. In conclusion, raptor gene expression is involved in mithramycin-mediated induction of erythroid differentiation of K562 cells and one of its mechanism of action is the inhibition of Sp1 binding to the raptor promoter.

Introduction

The mammalian target of rapamycin (mTOR) forms two complexes, named mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) which are regulated by phosphorylation, complex formation and localization within the cells [1–5]. mTORC1 is composed of mTOR, the regulatory associated protein of mTOR (raptor), mammalian LST8/G-protein β-subunit like protein (mLST8/GβL) and the recently identified partners PRAS40 and DEPTOR [6,7]. Raptor binds directly to mTOR signaling (TOS) motifs on downstream targets, including S6K1 (ribosomal S6 protein kinase 1) and 4EBP1 (eukaryotic initiation factor 4E-binding protein 1) as well as PRAS40 and Hif1α, thus linking them to the mTOR kinase [2,3]. mTORC1 senses and integrates diverse extra- and intracellular signals to promote anabolic and to inhibit catabolic cellular processes. This complex is characterized by the classic features of mTOR by functioning as a nutrient/energy/redox sensor and controlling protein synthesis [1,6]. The activity of this complex is stimulated by insulin, growth factors, serum, phosphatidic acid, amino acids (particularly leucine), and oxidative stress [6,8]. mTORC1 in yeast and mammals also promotes "ribosome biogenesis", a process whereby mTORC1 increases the transcription of ribosomal RNAs and proteins to augment cellular protein biosynthetic capacity [9–11]. mTOR Complex 2 (mTORC2) is composed of mTOR, rapamycin-insensitive companion of mTOR (Rictor), GβL, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1) [2,3,12,13]. mTORC2 has been shown to function

Abbreviations: Raptor, regulatory associated protein of mTOR; Rictor, rapamycin-insensitive companion of mTOR; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; m-TORC2, mTOR complex 2; Sp1, specific protein 1; MTH, mithramycin; RAPA, rapamycin; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; PBS, phosphate-buffered saline; TBS, tris-buffered saline; HbF, fetal hemoglobin; ODN, oligonucleotide.

* Corresponding author at: Department of Life Sciences and Biotechnology, Molecular Biology Section, University of Ferrara, Via Fossato di Mortara 74, 44121 Ferrara, Italy. Tel.: +39 532 974443; fax: +39 532 974500.
E-mail address: gam@unife.it (R. Gambari).

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as an important regulator of the cytoskeleton through its stimulation of F-actin stress fibers, paxillin, RhoA, Rac1, Cdc42, and protein kinase C α (PKCα) [14–17]. mTORC2 also appears to possess the activity of a previously elusive protein known as "PDK2." mTORC2 phosphorylates the serine/threonine protein kinase Akt/PKB at a serine residue S473. Phosphorylation of the serine stimulates Akt phosphorylation at a threonine T308 residue by PDK1 and leads to full Akt activation [13,16,17]; mTORC2 appears to be regulated by insulin, growth factors, serum, and nutrient levels [6,7]. Originally, mTORC2 was identified as a rapamycin-insensitive entity, as acute exposure to rapamycin did not affect mTORC2 activity or Akt phosphorylation. However, subsequent studies have shown that, at least in some cell lines, chronic exposure to rapamycin, while not affecting pre-existing mTORC2s, promotes rapamycin binding to free mTOR molecules, thus inhibiting the formation of new mTORC2 [14].

Despite the fact that the involvement of mTOR in all these and other biological processes has been firmly established, little information is available on the role of mTOR on erythroid differentiation. It has been demonstrated that rapamycin, an inhibitor of mTOR activity, is a potent inducer of erythroid differentiation of human leukemic K562 cells [18] and fetal hemoglobin production by β-thalassemic patients [19]. Accordingly, other inducers of K562 differentiation might operate through inhibition of mTOR [20–22]. In order to address this issue, mithramycin (MTH) was studied. This DNA-binding low molecular weight molecule is selective for G + C rich regions [23–25]. It binds to the minor groove of DNA generating unstable MTH-DNA complexes [20]. It is one of the most potent inducers of K562 differentiation [21] and HbF production by erythroid precursor cells from normal donors as well as β-thalassemia patients [26].

The main objective of the present study was to verify whether induction of differentiation by MTH is associated with inhibition of mTOR activity. The effects of MTH on raptor, rictor and mTOR gene expression were first analyzed by q-RT-PCR. Secondly, we determined by Western blotting, the production of mTOR, raptor and rictor proteins in MTH treated cells with the aim of determining whether mithramycin affects mTORC1, mTORC2 or both. Thirdly, we verified the possible effect of MTH on the interaction of the regulatory transcription factor Sp1 with the raptor promoter. This was approached by electrophoretic mobility shift assay, chromatin immunoprecipitation and treatment of target cells with Sp1 decoy molecules.

Materials and methods

Human K562 cell cultures

The human leukemia K562 [27,28] cells were cultured in a humidified atmosphere of 5% CO_2/air in RPMI 1640 medium (SIGMA, St. Louis, MO, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Biowest, Nuaille, F), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell growth was studied by determining the cell number per ml with a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA, USA).

Antibodies

The primary antibodies for Western blotting were: anti-Raptor cat. 2280, anti-Rictor cat. 2114, anti-mTOR cat. 2983, anti-p-mTOR (Ser2448) cat. 2971, anti-p-mTOR (Ser2481) cat.2974, anti-p70S6 kinase cat.2708, anti-p-p70S6 kinase (Thr389) cat.9234. All antibodies were purchase from Cell Signaling (Euroclone S.p.A., Pero, MI, Italy).

RNA extraction

Cells were isolated by centrifugation at 1500 rpm for 10 min at 4 °C, washed in PBS, lysed in Tri-reagentTM (Sigma–Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. The isolated RNA was washed once with cold 75% ethanol, dried and dissolved in diethylpyrocarbonate treated water before use.

Reverse transcription and quantitative real-time PCR (RT-qPCR)

The reagents for gene expression analysis by real-time RT-PCR were obtained from Applied Biosystems (Foster City, CA, USA). 500 ng of total RNA were reverse transcribed using random hexamers. RT-qPCR assay was carried out using gene-specific double fluorescently labeled probes. The primers and probes used to assay the expression of γ-globin, α-globin and mTOR mRNA were purchased from IDT (Integrated DNA technologies, San Jose, CA, USA). The nucleotide sequences used for real-time qPCR analysis of γ- and α-globin mRNAs and mTOR were as follows: α-globin forward primer, 5′-CAC GCG CAC AAG CTT CG-3′, α-globin reverse primer, 5′-AGG GTC ACC AGC AGG CAG T-3′, α-globin probe, 5′-FAM-TGG ACC CGG TCA ACT TCA AGC TCC T-TAMRA-3′; γ-globin forward primer, 5′-TGG CAA GAA GGT GCT GAC TTC-3′, γ-globin reverse primer, 5′-TCA CTC AGC TGG GCA AAG G-3′, γ-globin probe, 5′-FAM-TGG GAG ATG CCA TAA AGC ACC TGG-TAMRA-3′; mTOR forward primer 5′-GCT GTA CGT TCC TTC TCC TTC-3′, mTOR reverse primer 5′-CAA GAA CTC GCT GAT CCA AAT G-3′, mTOR probe, 5′-FAM-TGC ATT CCG-ZEN-ACC TTC TGC CTT CA-IABkFQ-3′. The nucleotide sequences used for real-time qPCR analysis of transferrin receptor and glycophorin A mRNAs were: transferrin receptor forward primer, 5′-TCA GAGCGTCGGGATATCG-3′, transferrin receptor reverse primer, 5′-TGA ACT GCC ACA CAG AAG AAC A-3′, transferrin receptor probe 5′-FAM-TGG CGG CTC GGG ACG GA-TAMRA-3′; glycophorin A forward primer, 5′-CGG TAT TCG CCG ACT GAT AAA-3′, glycophorin A reverse primer, 5′-AAA GGC AGT CTG TGT CAG GT-3′, glycophorin A probe, 5′-FAM-AAA GCC CAT CTG ATG TAA AAC CTC TTC CCC T-TAMRA-3′. The kit for quantitative RT-PCR for ζ-globin mRNA and ε-globin mRNA were from Applied Biosystems (ζ-globin mRNA: Hs00923579_m1; ε-globin mRNA: Hs00362216_m1). The primers and probes used to assay the expression of raptor mRNA (Assay ID Hs00977502_m1) and for Rictor (Assay ID Hs.PT.56a.40621153.g) were purchased from Applied Biosystems and from IDT, respectively. Relative expression was calculated using the comparative cycle threshold method and the endogenous controls human 18S rRNA (Assay ID 4310893E, Applied Biosystems) and RPL13A (Assay ID Hs03043885_g1, Applied Biosystems) as reference genes. Duplicate negative controls (no template cDNA) were also run with every experimental plate to assess specificity and to rule out contamination.

Western blotting

For extract preparation, MTH treated or untreated K562 cells were lysed in a ice cold RIPA buffer (10 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 150 mM NaCl, 1% NP40, 0.1% SDS, 5 mg/ml DeoxyCholic acid, 1 mM DTT, 2 mM PMSF, 2 mM Na_3VO_4, 10 mM NaF, 1 μg/ml Leupeptin, 1 μg/ml Aprotinin). Briefly, K562 cells (8×10^6 cells) were collected and washed twice with cold PBS (Phosphate-Buffered Saline, Lonza-Biowhittaker, Basel, Switzerland). Cellular pellets were then suspended with 400 μl of cold RIPA buffer, incubated on ice for 20 min and subjected to five cycles of freeze–thawing. Samples were finally centrifuged at 14,000 × *g* for 3 min at 4 °C and the supernatant cytoplasmic fractions were collected and immediately frozen at −80 °C. Protein concentration was determined

using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, USA). Ten μg of cytoplasmic extracts were denatured for 5 min at 98 °C in 1× SDS sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 50 mM Dithiotreithol (DTT), 0.01% bromophenol blue, 10% glicerol) and loaded on SDS-PAGE gel (10 cm × 8 cm) in Tris-glycine Buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). A biotinylated protein ladder (size range of 9–200 kDa) (Cat. 7071, Cell Signaling, Euroclone S.p.A., Pero, MI, Italy) and/or a prestained multicolor protein ladder (size range 10–260 kDa) (Cat 26634, Thermo Fisher Scientific, Rockford, USA) were used as standards to determine molecular weight. The electrotransfer to 0.2 μm pore size nitrocellulose membrane (Pierce, Euroclone S.p.A., Pero, Milano, Italy) was performed over-night at 360 mA and 4 °C in electrotransfer buffer (25 mM Tris, 192 mM Glycine, 5% methanol). The membranes were prestained with Ponceau S Solution (Sigma, St. Louis, MO, USA) to verify the transfer, washed with 25 ml TBS (10 mM Tris–HCl pH 7.4, 150 mM NaCl) for 10 min at room temperature and incubated in 25 ml of blocking buffer for 2 h at room temperature. The membranes were washed three times for 5 min each with 25 ml of TBS/T (TBS, 0.1% Tween-20) and incubated with the primary rabbit monoclonal antibody (1:1000) in 15 ml primary antibody dilution buffer with gentle shaking over-night at 4 °C. The next day, the membranes were washed three times for 5 min each with 20 ml of TBS/T and incubated in 15 ml of blocking buffer, with gentle shaking for 2 h at room temperature, with an appropriate HRP-conjugated secondary antibody (1:2000) and an HRP-conjugated anti-biotin antibody (1:1000) used to detect biotinylated protein marker. Finally, after three washes each with 20 ml of TBS/T for 5 min, the membranes were incubated with 10 ml LumiGLO® (0.5 ml 20x LumiGLO®, 0.5 ml 20× Peroxide and 9.0 ml Milli-Q water) (Cell Signaling, Euroclone S.p.A., Pero, MI, Italy) with gentle shaking for 5 min at room temperature and exposed to x-ray film (Pierce, Euroclone S.p.A., Pero, MI, Italy). In order to re-probe the membranes, they were stripped using the Restore™ Western Blot Stripping Buffer (Pierce, Euroclone S.p.A., Pero, MI, Italy) and incubated with other primary and secondary antibodies. The chemiluminescent signal was visualized on X-ray films and the intensity of the immunopositive bands was analyzed by Gel Doc 2000 (Bio-Rad Laboratoires, MI, Italy) using Quantity One program to elaborate the intensity data of our specific target protein.

Preparation of nuclear extracts for bandshift and supershift assays

Nuclear extracts were prepared as described by Andrews and Faller [29]. Briefly, cells were collected, washed twice with ice-cold phosphate-buffered saline, and suspended in 0.4 ml/10^7 cells of hypotonic lysis buffer (10 mM Hepes/KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.2 mM phenylmethanesulfonyl fluoride). After incubation on ice for 10 min, the mixture was vortexed for 10 s, and nuclei were pelleted by centrifugation at 12,000 × *g* for 10 s, then nuclear proteins were extracted by incubation of the nuclei for 20 min on ice with intermittent gentle vortexing in 20 mM Hepes/KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethanesulfonyl fluoride, 1 μg mL^{-1} aprotinin, 1 μg mL^{-1} leupeptin, 2 mM Na_3VO_4, and 10 mM NaF (Sigma, St Louis, MO, USA); cell debris was removed by centrifugation at 12,000 × *g* for 5 min at 4 °C. The BCA method was used to measure the protein concentration in the extract, which was then stored in aliquots at −80 °C.

Electrophoretic mobility shift assays (EMSA)

The double-stranded oligonucleotides (ODN) used in the EMSA are reported in Table 1 [30]. 3 pmol of ODN were ^{32}P-labeled using OptiKinase (GE Healthcare, Chalfont St Giles, UK), annealed to an excess of complementary ODN and purified from [γ-^{32}P]ATP (Perkin Elmer, Wellesley, MA, USA). Binding reactions were performed by incubating 2 μg of nuclear extract and 16 fmol of ^{32}P-labeled double-stranded ODN, with or without competitor in a final volume of 20 μL of binding buffer (20 mM Tris–HCl, pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 0.01% TritonX100, 0.05 μg μL^{-1} of poly dI-dC, 0.05 μg μL^{-1} of a single-stranded ODN) [31]. Competitor (100 fold excess of unlabeled ODNs) and nuclear extract mixture were incubated for 15 min and then probe was added to the reaction. After a further incubation of 30 min at room temperature samples were immediately loaded onto a 6% nondenaturing polyacrylamide gel containing 0.25 × Tris/borate/EDTA (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM EDTA, pH 8) buffer. Electrophoresis was carried out at 200 V. Gels were vacuum heat-dried and subjected to autoradiography. To verify the effects of mithramycin, DNA probes or nuclear extracts, were preincubated for 1 h at 4 °C with different concentration of the compound before the EMSA incubation. Supershift assays were performed as described previously [31,32] by using 2 μg of commercially available antibodies specific for Sp1 (cat.07-645) transcription factor and anti-NF-kB (cat. 06–556) as a control unrelated antibody (Upstate Biotechnology Inc., Lake Placid, NY, USA).

Bio-Plex analysis

The levels of multiple transcription factors within nuclear extracts obtained from stimulated cells were analyzed using the Bio-Plex technology (Bio-Rad Laboratories, Hercules, CA) and the BioSource's Transcription Factor (TF) Assays (Biosource International, Inc., California USA). Nuclear extracts were prepared using BioSource's Nuclear Extraction Kit (Biosource International, Inc., California USA) and quantified using the Bradford assay. The assay was performed as reported by the manufacturer. Briefly, biotin labeled DNA probes and controls or nuclear extract samples were incubated for 20 min at 25 °C in 96 well PCR thermocycler-compatible microtiter plate. Subsequently Digestion Reagent containing nuclease was added to samples (except positive control) for 20 min at 37 °C. The fluorescently encoded microspheres conjugated to DNA sequences complementary to the probes were then incubated with samples for 45 min at room temperature. Finally, the individual mixtures were transferred to the wells of a filter plate, washed and incubated with streptavidin-RPE. After the washing step the samples were analyzed in Bio-plex instrument.

Transcription factor decoy (TFD) approach targeting Sp1 protein binding on raptor promoter

The effect of the Sp1e consensus oligonucleotide decoy [33,34] on raptor transcription was evaluated by adding 2 μg/ml of *Sp1e* double-stranded oligonucleotide or a scrambled sequence of the same length as a control (*scramble ODN*) (Table 1), and 4 μg/ml μg/ml of lipofectamine 2000 to K562 cells seeded at 50–70% confluence in 16 mm wells. Twenty-four hours later, RNA extraction and reverse transcription were performed. Aliquots, 1/20 μl of cDNA were used for each SYBR Green realtime PCR reaction to quantify the depletion of raptor transcripts, using the *Raptor F* primer and the *Raptor R* reverse primer (Table 1) designed to amplify a 355 bp sequence. Amplification of human GAPDH cDNA served as an internal standard (housekeeping gene). Real-time PCR reactions were performed for a total of 40 cycles (95 °C for 3 min, 66 °C for 30 s, and 72 °C for 25 s). The ΔΔCT method was used to compare gene expression data.

Table 1
Double stranded syntetic oligonucleotides and PCR primers employed.

Name	Method	Amplified region	Sequences
Sp1mer[a]	EMSA	N.A.[b]	5′CCCTGGCCACGCCTCACTG3′
Sp1a	EMSA	N.A.	5′TGCACCACCACGCCTGGCCTCG3′
Sp1b	EMSA	N.A.	5′CACGCCACCACGCCCAGCTAAT3′
Sp1c	EMSA	N.A.	5′CGCACCACCACGCCCAGCTAAT3′
Sp1d	EMSA	N.A.	5′TTAGTAGGGACGGGGTTTCACC3′
Sp1e	EMSA and decoy	N.A.	5′TTTAATAACACGCCTCTACTGA3′
Sp1f	EMSA	N.A.	5′AGGTAACGGGGTCGGGGACTCTTTC3′
Sp1g	EMSA	N.A.	5′CTGTCGGCCACGCCGTAGGCCG3′
Asp ODN	EMSA	N.A.	5′TGTCGAATGCAATCACTAGAA3′
Scramble ODN	Decoy	N.A.	5′AGTCGTCACGTAAGTCGAGCAC3′
Raptor F	Decoy real time PCR	Raptor transcripts	5′CTGCAAGCATTCCAGGTGTG3′
Raptor R	Decoy real time PCR	Raptor transcripts	5′GTTCAGCTGGCATGTAGGGG3′
GAPDH F[c]	Decoy real time PCR	GAPDH transcripts	5′AAGGTCGGAGTCAACGGATTT3′
GAPDH R	Decoy real time PCR	GAPDH transcripts	5′ACTGTGGTCATGAGTCCTTCCA3′
Raptor ChIP F	ChIP real time PCR	Raptor promoter	5′AACCGACAGTTTCATTTGTAGGATTG3′
Raptor ChIP R	ChIP real time PCR	Raptor promoter	5′AAACTCATCTCATCAGCCCATCA3′
Neg ChIP F	ChIP real time PCR	Negative control region	5′AGACAGGGTTTCACCATGTTGG3′
Neg ChIP R	ChIP real time PCR	Negative control region	5′GCCATAGCTAACTGCAGAGGACA3′

[a] Sp1 consensus sequence [30].
[b] N.A., not applicable.
[c] GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

Chromatin Immunoprecipitation Assays (ChIP)

Chromatin immunoprecipitation assays were performed using the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology) [31,35]. Briefly, a total of 6×10^7 K562 cells, untreated or treated with MTH for 24 h, were incubated, for 10 min at room temperature, with 1% formaldehyde culture medium. After washing in phosphate-buffered saline, glycine was added to a final concentration of 0.125 M. The cells were then suspended in 1.5 ml of lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris–Cl, pH 8.1) plus protease inhibitors (1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) and the chromatin subjected to sonication (using a Sonics Vibracell VC130 sonicator with a 2-mm probe). Fifteen 15-s sonication pulses at 30% amplitude were required to shear chromatin to 200–1000 bp fragments. 0.2-ml aliquots of chromatin were diluted to 2 ml in ChIP dilution buffer containing protease inhibitors and then cleared with 75 μl of salmon sperm DNA/protein A-agarose 50% gel slurry (Upstate Biotechnology) for 1 h at 4 °C before incubation on a rocking platform with either 6–10 μg of specific antiserum Sp1 (Upstate Biotechnology, cat.07-645) or normal rabbit serum. 20 μl of diluted chromatin was saved and stored for subsequent PCR analysis as 1% of the input extract). Incubations occurred overnight at 4 °C and continued an additional 1 h after the addition of 60 μl protein A-agarose slurry. Thereafter the agarose pellets were washed consecutively with low salt, high salt and LiCl buffers. DNA/protein complexes were recovered from the pellets with elution buffer (0.1 M NaHCO3 with 1% SDS), and cross-links were reversed by incubating overnight at 65 °C with 0.2 M NaCl. The samples were treated with RNase A and proteinase K, extracted with phenol/chloroform and ethanol-precipitated. The pelletted DNAs were washed with 70% ethanol and dissolved in 40 μl of Tris/EDTA. 2 μl aliquots were used for each real-time PCR reaction to quantitate immunoprecipitated promoter fragments.

Real-time PCR quantification of immunoprecipitated promoter fragments

For quantitative real-time PCR reaction conditions each 25 μl reaction contained 2 μl of template DNA (from chromatin immunoprecipitations), 10 pmol of primers (Table 1) and 1× iQ™ SYBR® Green Supermix (Bio-Rad). Real-time PCR reactions were performed for a total of 40 cycles (97 °C for 15 s, 65 °C for 30 s, and 72 °C for 30 s) using an iCycler IQ® (Bio-Rad). The relative proportions of immunoprecipitated promoter fragments were determined based on the threshold cycle (*Tc*) value for each PCR reaction. Real-time PCR data analysis followed the methodology previously described [35–37]. A ΔTC value was calculated for each sample by subtracting the *Tc* value for the input (to account for differences in amplification efficiencies and DNA quantities before immunoprecipitation) from the *Tc* value obtained for the immunoprecipitated sample. Real-time PCR data analyses were obtained using the comparative *Ct* method: a Δ *Ct* value was calculated for each sample by subtracting the *Ct* value for the sample amplified with raptor promoter primers from the *Ct* value obtained for the same sample amplified with negative control primers. For each kind of immunoprecipitation (IgG or Sp1 antiserum), a $\Delta\Delta$ *Ct* value was then calculated by subtracting the Δ *Ct* value for the untreated cells sample from the ΔCt value for the treated cell samples. Fold differences were then determined by the $2^{-\Delta\Delta Ct}$ method. Each sample was quantified in duplicate for at least three separate experiments. Mean ± SD values were determined.

Cell cycle analysis

For flow cytometric analysis of DNA content, 1×10^6 K562 cells in exponential growth were treated with IC_{75} concentrations of the tested compounds. After 72 h, the cells were centrifuged, washed once with PBS, then treated with lysis buffer containing RNAse A, NP40 0,1%, and finally stained with propidium iodide at 50 μg/ml (DNA QC particles, Becton Dickinson, Milan, Italy). Samples were analyzed on a Becton Coulter Epics XL-MCL flow cytometer. For cell cycle analysis, DNA histograms were analyzed using MultiCycle® for Windows (Phoenix Flow Systems, San Diego, CA).

Statistical analysis

All the data were normally distributed and presented as mean ± SD. Statistical differences between groups were compared using one-way ANOVA (ANalyses Of VAriance between groups) software. *P* values were obtained using the Paired t test of the GraphPad Prism Software. Statistical differences were considered significant when $^{*}p < 0.05$, highly significant when $^{**}p < 0.01$.

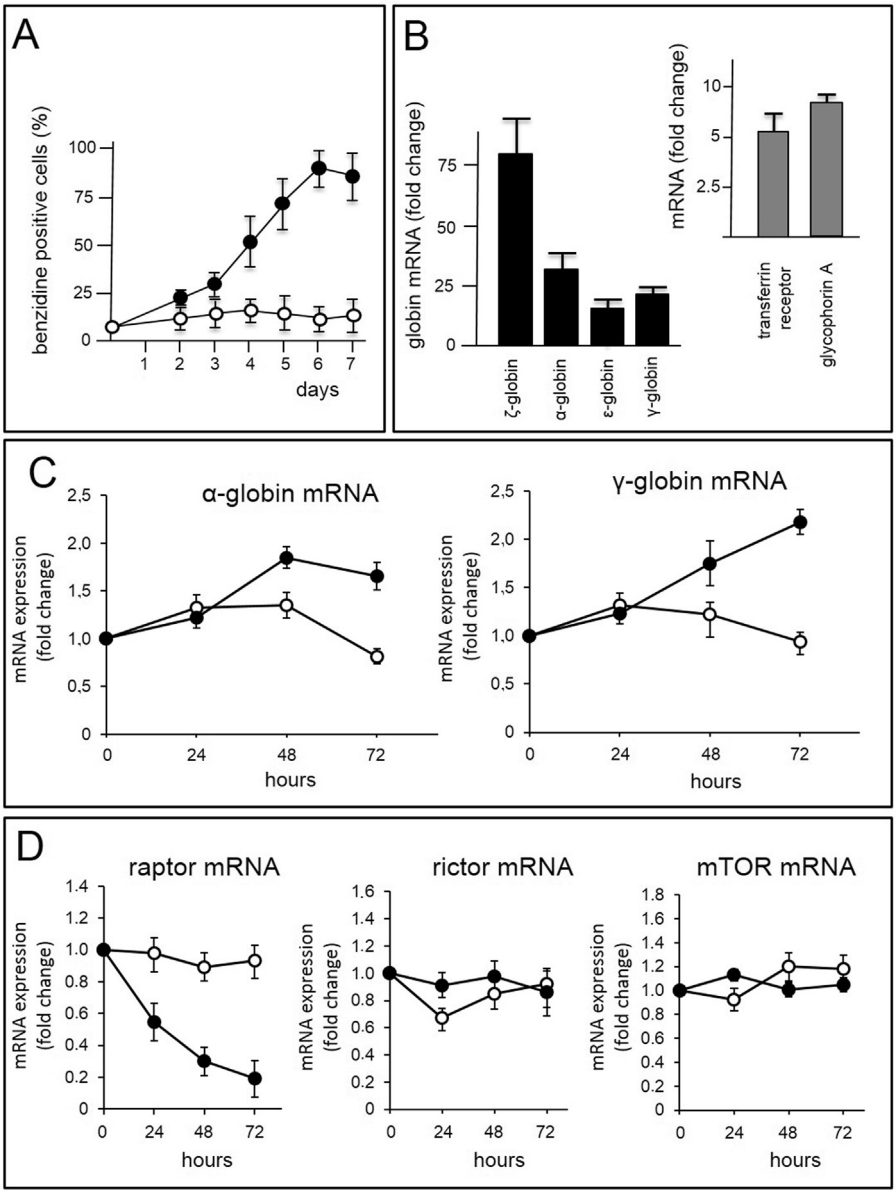

Fig. 1. Effects of mithramycin (MTH) on erythroid induction and gene expression of K562 cells. A,B. Effects of 30 nM mithramycin on kinetics of the increase of the proportion of benzidine-positive cells (%) (average ± SD; number of independent experiments: 5) (A) and on the expression of ζ-globin, α-globin, ε-globin, γ-globin, transferrin receptor and glycophorin A genes evaluated by RT-qPCR of RNA isolated from K562 cells treated with 30 nM mithramycin for 5 days (B). MTH-treated samples of panel A are indicated with black circles). C,D. Effects of MTH on (A) α-globin and γ-globin and (B) raptor, rictor and mTOR gene expression in MTH-treated K562 cells. RNA samples were obtained from K562 cells cultured in the absence (white circles) or in the presence (black circles) of 30 nM MTH for the indicated length of time. The data shown in panels B-D represent changes in gene expression referred to time 0, determined by quantitative RT-PCR (average ± SD; $n = 5$).

Results

Treatment of K562 cells with MTH leads to inhibition of raptor mRNA accumulation, but not of rictor and mTOR mRNAs

To relate globin gene expression to that of genes involved in m-TORC1 and m-TORC2 pathways, mRNAs were studied by quantitative RT-PCR analysis using as template cytoplasmic RNA isolated from K562 cells cultured for different length of time in the absence or in the presence of 30 nM MTH. In order to determine the extent of erythroid induction, the proportion of benzidine-positive cells was firstly measured (Fig. 1A) and was shown to be increased from 1–5% (in control untreated cells) to 75–85% (in MTH-treated cells), in agreement with previously published observations [26]. Fig. 1B (left side of the panel) shows that MTH treatment induces, after 5 days treatment, a large increase of (a) the α-like ζ-globin and α-globin mRNAs and (b) the β-like ε-globin and γ-globin mRNAs. These data confirm that the MTH mediated erythroid induction was fully activated, sustaining the strong effect of MTH as erythroid inducer of this cell line. The ongoing of the erythroid program in MTH-induced K562 cells is also confirmed by the increase of other erythroid associated markers, including those encoded by the CPOX (coproporphyrinogen oxidase), UROS (uroporphyrinogen III synthase), UROD (uroporphyrinogen decarboxylase), FECH (ferrochelatase), NFE2L3 nuclear factor erythroid-derived 2-like 3), EPB49 (erythrocyte membrane protein band 4.9), SLC4A1 (solute carrier family 4, anion exchanger, member 1 erythrocyte membrane protein band 3, Diego blood group) mRNAs (data not shown) and transferrin receptor and glycophorin A mRNAs (Fig. 1B, right side of the panel). Possible early changes in expression of genes

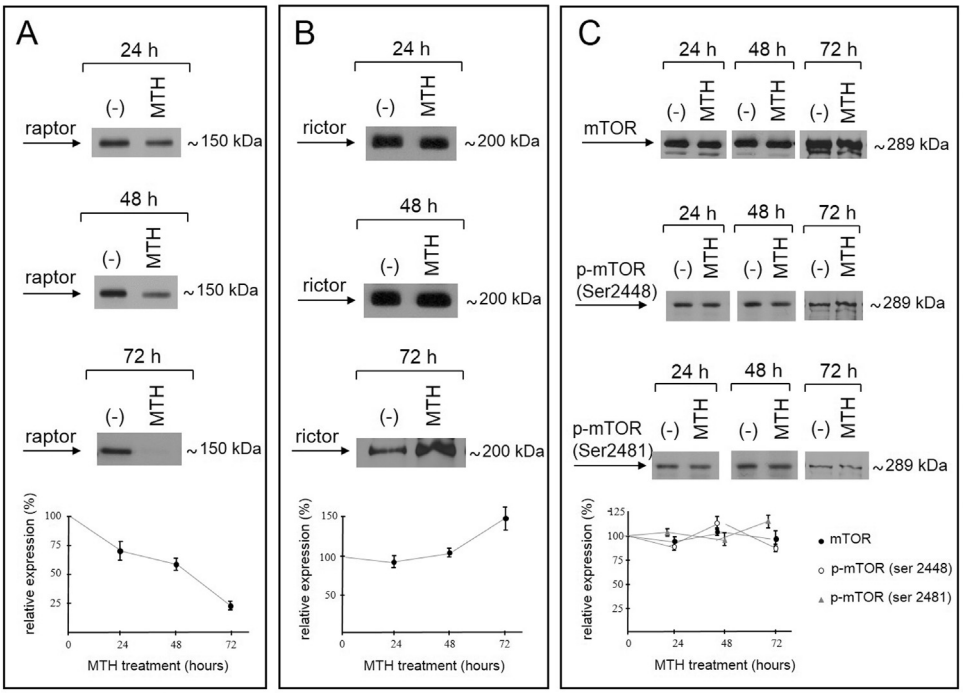

Fig. 2. MTH effects on raptor, rictor and mTOR in K562 cells. Western blotting analyses were performed on protein extracts obtained from K562 cells treated with 30 nM MTH for the indicated length of time, using raptor (A), rictor (B) and mTOR (C, upper panel), p-mTOR (Ser2448) (C, middle panel), p-mTOR (Ser2481) (C, lower panel) monoclonal antibodies. The relative expression values of samples from MTH treated with respect to untreated cells were obtained from densitometric measurement and reported in the lower part of the panels. Data represent the average ± SD; $n = 3$).

participating to the mTOR pathway, such as raptor, rictor and mTOR, were then performed using RT-PCR analyses. Panel C of Fig. 1 shows the early increase of the levels of α-globin and γ-globin mRNAs. Fig. 1D shows that (a) the content of raptor mRNA sharply decreases following MTH treatment (left side of the panel) and (b) that this decrease occurs before the MTH-induced increase of globin mRNAs (see Fig. 1, panel C). On the contrary, no inhibition of rictor mRNA and mTOR mRNA was detected (Fig. 1D, middle and right sides of the panel). These findings were fully supported by the Western blotting analysis of cytoplasmic extracts isolated from K562 cells cultured for 24, 48 and 72 h with MTH, as reported in Fig. 2. The results shown in Fig. 2A demonstrate a sharp inhibition of raptor protein production following MTH treatment. As far as rictor expression is concerned, no changes in its production were observed at 24 and 48 h, while after 72 h of treatment, a slight increase was detectable (Fig. 2B). In addition, no changes in mTOR content (Fig. 2C, upper part of the panel) was found in MTH treated cells. Finally, no major changes were found in mTOR phosphorylation at Ser 2448 and Ser 2481 (Fig. 2C, middle and lower parts of the panel).

These data demonstrate for the first time strong inhibitory effects of mithramycin on mTORC1, but not on mTORC2. Accordingly, the possible effects of mithramycin on the raptor gene promoter were determined.

Structure of the raptor gene promoter

In order to identify putative regulatory regions located within the raptor gene promoter, computer-aided analysis of the raptor promoter sequence was performed with the aim to identify putative binding sites for transcription factors (Fig. 3A). Within the −1400/+1 promoter sequence, we identified homology to the following transcription factor binding sites: LyF-1, AP1, C/EBP, USF, Oct, GATA-1 and at least twelve sequences 98–81% homologous to the canonical 5′-CCT GGC CAC GCC TCA CTG-3′ Sp1 binding site. Mithramycin has been widely reported as an antibiotic that binds DNA exhibiting selective interactions with G + C rich regions, demonstrated by computer modeling [38], Biacore analysis [20], and DNase Footprinting [21]. As MTH was expected to affect Sp1-DNA interactions as already reported [39–41], the *in vitro* interactions of Sp1 with the raptor Sp1 binding sites present within the raptor gene promoter were analyzed.

In vitro interaction of Sp1 transcription factor with the Sp1 binding sites of the raptor gene promoter: effects of MTH

To determine whether the raptor Sp1 binding sites are able to interact with the Sp1 nuclear transcription factor, EMSA experiments were carried out using 3 μg of K562 nuclear extracts and the raptor-Sp1 probes described in Fig. 3A and Table 1. The results obtained are reported in Fig. 3B–E and indicate that the Sp1 probes interact differently with K562 nuclear extracts. The specific complexes generated by the interaction between nuclear extracts and *Sp1b*, *Sp1c*, and *Sp1e are* arrowed in panels B-E of Fig. 3. The consensus Sp1*mer* probes efficiently compete with the binding of nuclear extracts to the consensus ^{32}P-labeled *Sp1mer* probe (Fig. 3B), while *Sp1a*, *Sp1d*, *Sp1f* and *Sp1g* were less efficient in binding. Fig. 3C shows a preliminary experiment demonstrating that *Sp1e* competes, as *Sp1mer*, for Sp1/DNA interactions. Fig. 3D shows that *Sp1e* is efficient in competing with the binding of nuclear extracts to the consensus ^{32}P-labeled *Sp1mer* probe. Fig. 3E shows that the binding activity of *Sp1e* occurs also when purified Sp1 protein is employed. Similar results were obtained with *Sp1b* and *Sp1c* oligonucleotides (data not shown).

Sp1 transcription factor is involved in raptor gene expression

To determine whether the Sp1 transcription factor might be involved in raptor gene expression we determined the extent of Sp1 content in protein extracts from K562 cells using Bio-Plex

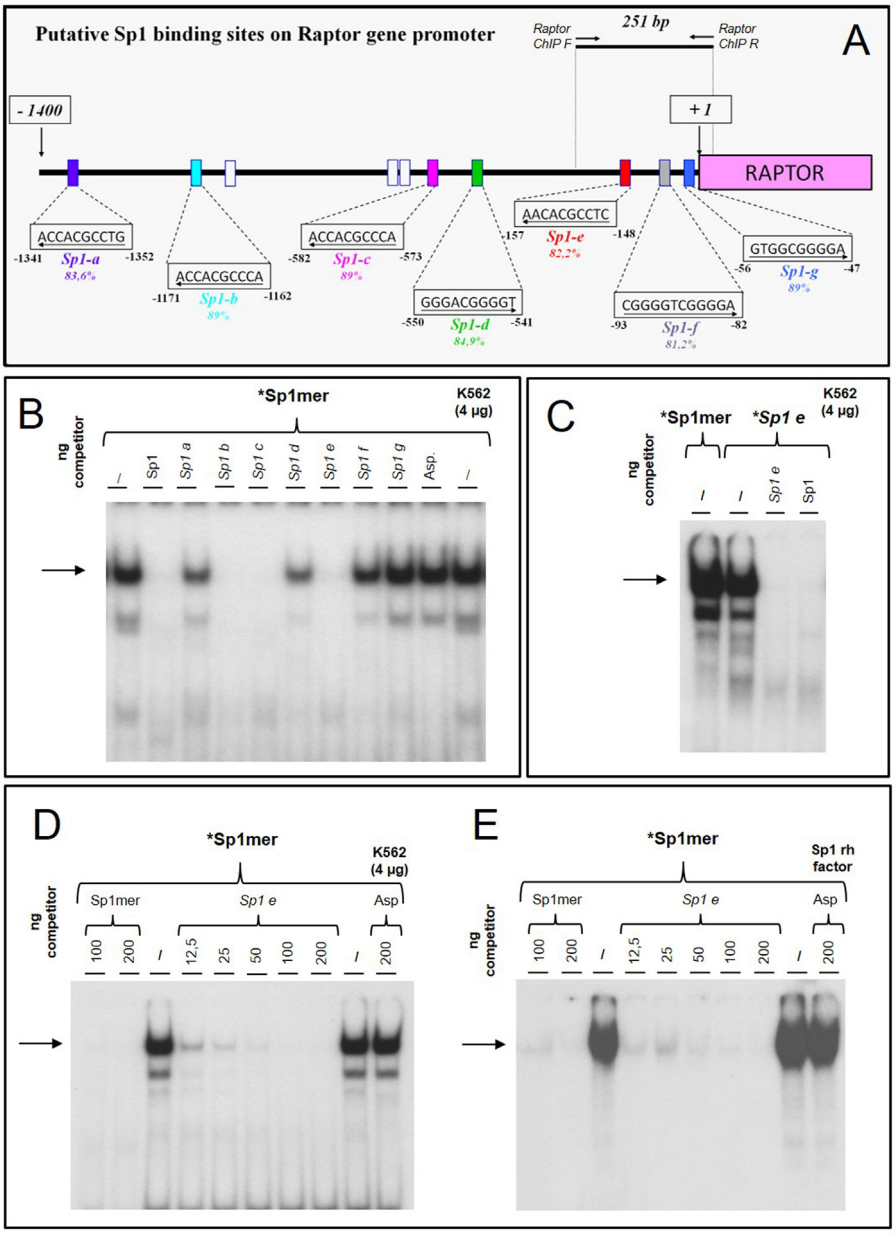

Fig. 3. Schematic representation of raptor gene promoter and comparison between the binding efficiency of three Sp1 consensus sites. (A) Boxes indicate the Sp1 homology binding sites. Nucleotide position and sequences (only for sites displaying homology ≥ 81%) are indicated. The length and position of the promoter fragment amplified in ChIP PCR reaction are also reported together with the location of the Raptor ChIP F and Raptor ChIP R PCR primers. (B-E) Competitive bandshift assays performed using the Sp1 consensus binding site *Sp1mer* (B, C, D, E) and *Sp1e* (C) oligonucleotide as probes, nuclear extracts from K562 cells (B, C, D) or recombinant Sp1 factor (E), and increasing quantity of the competitor oligonucleotides as indicated.

assays. Fig. 4A shows high content of Sp1 in K562 cells. It should be pointed out however, that we have no evidence for the down regulation of Sp1 in MTH-treated K562 cells (Fig. 4B). In order to obtain results clarifying a possible role of Sp1 in the regulation of raptor gene expression a decoy experiment was performed, using a previously published protocol [34]. The results obtained show that the decoy treatment using double stranded *Sp1e* oligonucleotide induces decrease of raptor mRNA content, suggesting a role of Sp1 in the control of raptor gene expression.

Effect of MTH on in vitro interaction of Sp1 transcription factor with the Sp1 binding sites of the raptor gene promoter

Fig. 5 demonstrates that interaction of MTH with *Sp1e* prevents binding of nuclear extracts to the molecule (Fig. 5A, right side of the panel). In addition, Fig. 5B (right side of the panel) shows that the inhibitory effects of MTH occur also on pre-formed Sp1-DNA complexes. Both these MTH-mediated effects were similar to those obtained using Sp1mer (Fig. 5A and B, left side of the panels). These data clearly indicate that MTH is able to inhibit the *de novo* interaction of the Sp1 transcription factor within target Sp1 sequences, as well as to disassemble preformed Sp1/DNA complexes. MTH treatment of K562 cells may therefore lead to sharp decreases of Sp1 transcription factor occupancy at the level of the raptor gene promoter. In order to conclusively demonstrate that the transcription factor Sp1 binds to *Sp1e* sequence, supershift experiments were performed using monoclonal antibodies against Sp1 and nuclear extracts from K562 cells. The addition of the antibody induces a clear supershift (indicated in Fig. 5C with an arrowhead) showing that Sp1 nuclear factor binds *in vitro* to the *Sp1e* probe. An antibody

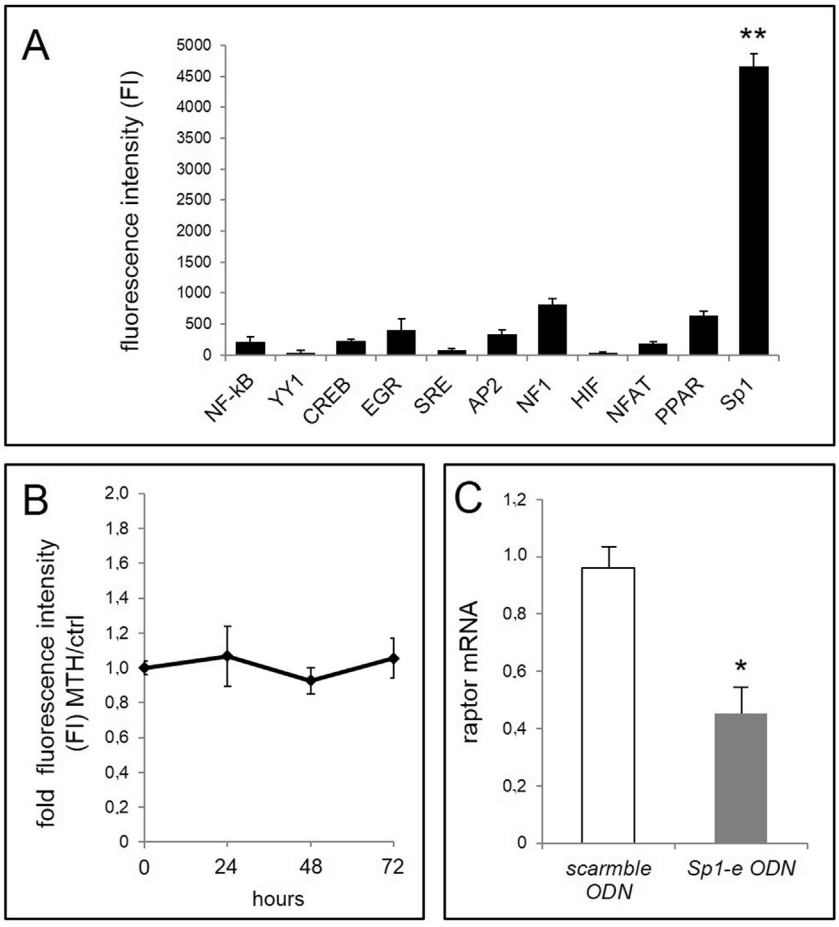

Fig. 4. Sp1 expression in K562 cells and effects of decoy molecules targeting Sp1 transcription factors. (A) Bio-Plex analysis of multiple transcription factors within nuclear extracts (expressed as fluorescence intensity) obtained from untreated K562 cells. (B) Level of Sp1 transcription factor within nuclear extracts obtained from MTH treated K562 cells for 0, 24, 48 and 72 h by Bio-plex technology. Data represent the average ± SD. ($n = 3$). (C) Effect of the *Sp1e* "decoy" ODN (gray box) or a scrambled unrelated oligonucleotide (white box), on raptor mRNA levels. The cDNA obtained from total RNA was subjected to quantitative real-time RT-qPCR for the raptor specific transcript. Relative quantification was calculated by the ΔΔCt method using untreated cells as control sample (value = 1). Data represent the average ± SD of triplicate experiments. p values were obtained using the Paired t test of the GraphPad Prism Software. Statistical significance: $^{*}p < 0.05$, significant; $^{**}p < 0.01$, highly significant.

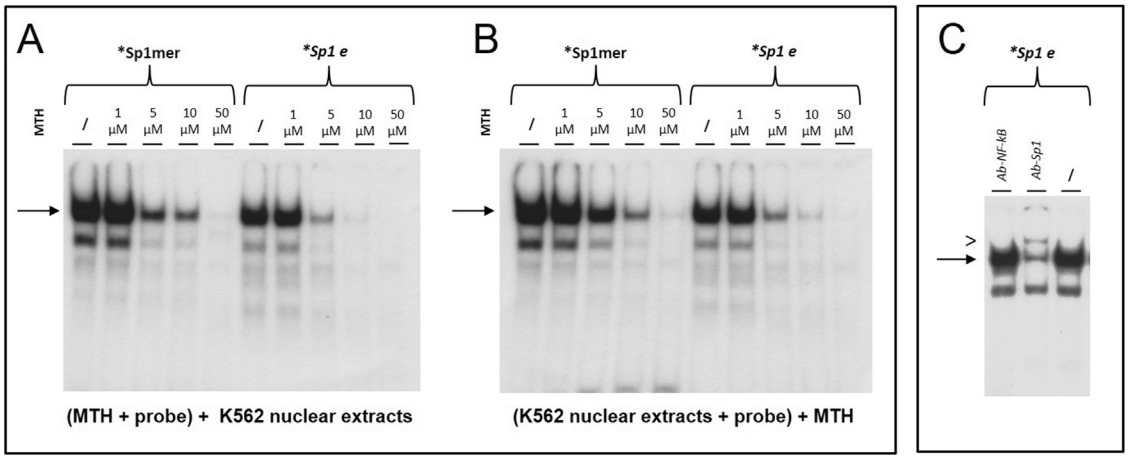

Fig. 5. MTH interferes with the interactions between Sp1 transcription factor and Sp1-sites of the raptor promoter sequences. (A,B) Bandshift assay was performed (A) pre-incubating the probes with the indicated concentrations of MTH, then adding nuclear extracts or (B) pre-incubating the probes with nuclear extracts, then adding the indicated increasing concentrations of MTH. (C) Supershift assay was performed using *Sp1e* as probe and 4 μg of nuclear extract from K562 cells; the probe was incubated with nuclear extracts in the absence (/) of antibody or in the presence of antibodies against Sp1 (Ab-Sp1) or NF-kB (Ab-NF-kB) factors. Arrows indicate the specific complexes and arrowhead indicates the super-shifted complexes.

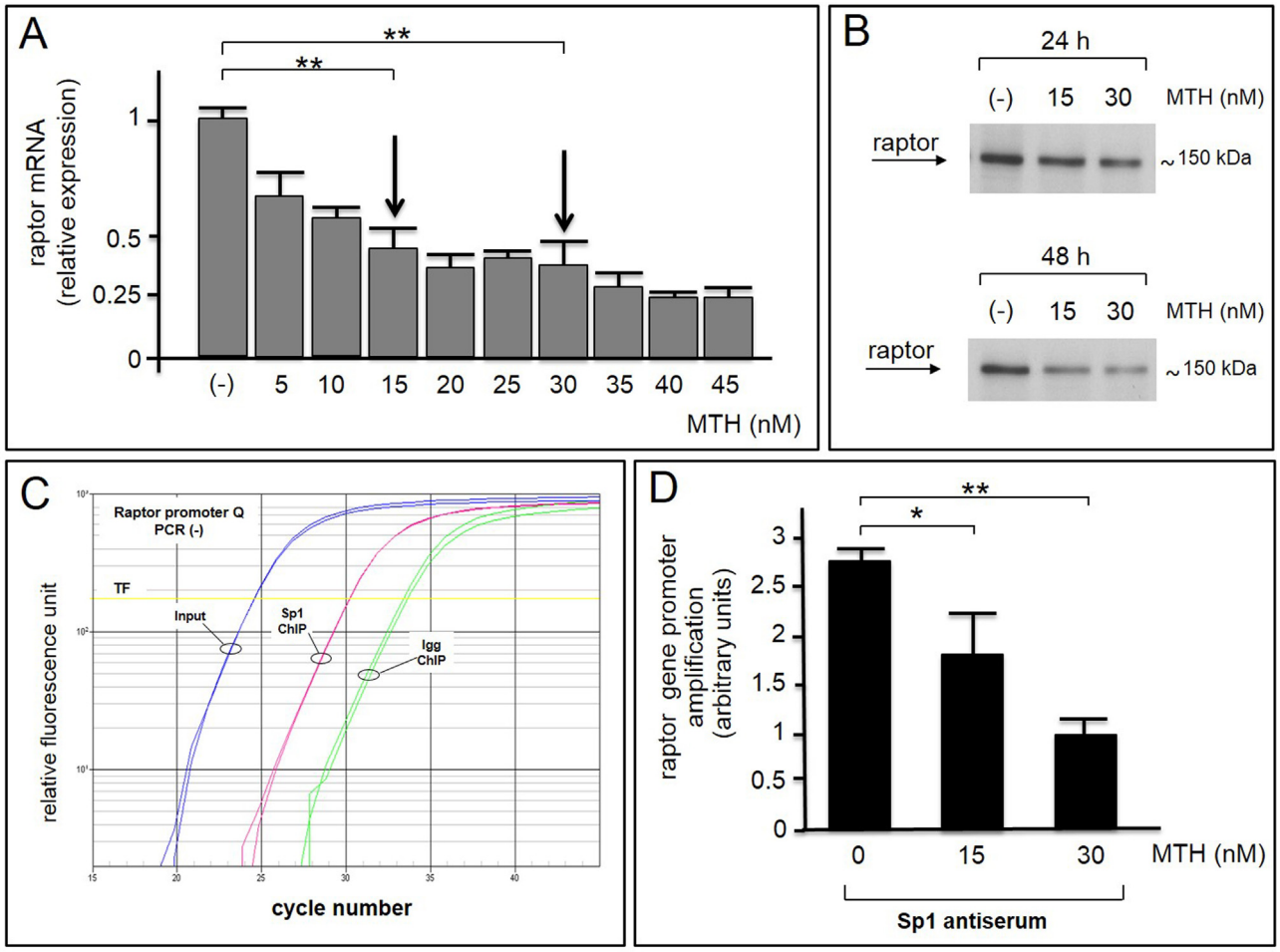

Fig. 6. MTH inhibits in K562 cells the recruitment of Sp1 to the raptor gene promoter. (A,B) Characterization of the MTH-mediated effects on raptor gene expression analyzed by RT-qPCR (A) and by Western blotting (B). K562 cells were cultured for 24 h with the indicated concentrations of MTH for RT-qPCR, while protein expression was analyzed in extracts from K562 cells cultured with 15 and 30 nM MTH for 24 and 48 h, as indicated. (C) Quantitative real-time PCR profiles for the amplification of raptor promoter from a representative ChIP assay in which chromatin from K562 cells was immunoprecipitated using Sp1 antiserum. Input, genomic DNA without antibodies addition; Sp1ChIP, ChIP performed with the Sp1 monoclonal antibody; Igg ChIP, control ChIP with non-immune serum. The data demonstrate the early exponential increase in fluorescence as a result of SYBR Green I incorporation into the amplifying raptor promoter fragment. (D) *In vivo* association of Sp1 transcription factor with raptor promoter obtained from ChIP experiment on K562 cells treated with 15 and 30 nM MTH. The results, obtained from ChIP assay quantitative real-time PCR using negative control IgG and Sp1 antiserum, were analyzed following the methodology described in Section 'Materials and methods'. The fold decrease compares the values obtained by raptor gene promoter amplification of untreated K562 cells and cells treated with 15 or 30 nM mythramicin. Data reported in panels A and D represent the average ± SD of triplicate experiments. *p* values were obtained using the Paired t test of the GraphPad Prism Software. Statistical significance: $^{*}p < 0.05$, significant; $^{**}p < 0.01$, highly significant.

against NF-kB failed to induce supershift (Fig. 5C, left row). Chromatin immunoprecipitation was performed to verify the effects of MTH on this binding activity in an *in vivo* context.

Chromatin immunoprecipitation (ChIP) analysis

Chromatin immunoprecipitation (ChIP) assays were performed on K562 cells either untreated, or treated with MTH to characterize the MTH effects on Sp1 function in intact cells. An RT-qPCR based study of the effects on raptor gene expression of different MTH concentrations (Fig. 6A) was conducted prior to performing ChIP. This treatment was performed for 24 h and demonstrated a concentration dependent inhibitory activity of MTH, suggesting that 15 nM and 30 nM concentrations might be suitable for ChIP assay. In parallel, the effects of 15 nM and 30 nM MTH treatments on raptor production were assessed using Western blotting on protein extracts isolated after 24 and 48 h from MTH-treated cells (Fig. 6B). ChiP was performed on K562 cells treated for 24 h with 15 nM and 30 nM MTH. Chromatin was immunoprecipitated using Sp1 antiserum and quantitative amplification of raptor gene promoter was performed on purified DNA. A representative ChIP assay is shown in Fig. 6C, in which it is clearly evident that amplification curves from samples immunoprecipitated with Sp1 antisera (Sp1 ChIP) reach threshold 5 cycles early than those treated with non immune serum (Igg ChIP). Fig. 6D shows that raptor-specific PCR is less efficient when samples from MTH-treated cells are employed, suggesting that MTH dose-dependently inhibits the recruitment of Sp1 to the raptor gene promoter.

MTH treatment is associated with inhibition of mTORC1 regulated biological process

In order to obtain a final proof of principle sustaining that the raptor/mTOR network is involved in MTH effects, biological parameters downstream from mTOR were considered [42–46]. K562 cells were treated with 30 nM MTH and the expression levels of mTORC1-regulated p70S6 kinase analyzed. The results are shown in Fig. 7A, and clearly sustain the concept that MTH inhibits the phosphorylation of p70S6 kinase. Fig. 7B shows the involvement of p70S6 kinase on the cell cycle [43,45], and demonstrates that MTH treatment of K562 cells leads to an increase of G1-phase cells,

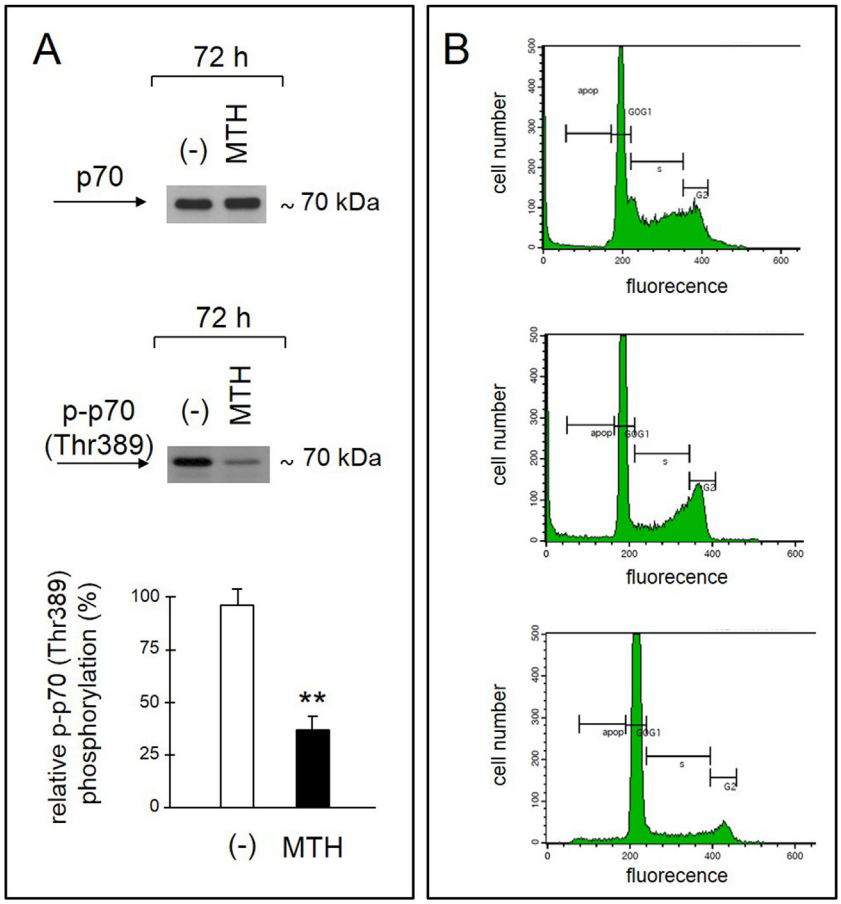

Fig. 7. Effects of MTH on p70S6 kinase and cell cycle. (A) Western blotting analysis was performed on protein extracts obtained from K562 cells treated with 30 nM MTH for the indicated length of time, using p70 and p-p70 (Thr389) monoclonal antibody. Densitometric values indicating the relative p-p70 (Thr389) phosphorylation are reported in the lower part of panel A. (B) Representative histograms of flow cytometry data of untreated control K562 cells (upper panel), K562 cells treated for 3 days (middle panel) or 4 days (lower panel) with 30 nM MTH (IC50). After 3 or 4 days of incubation the cells were labeled with propidium iodide and analyzed by flow cytometry as described in Section 'Materials and Methods'. Data reported in panel A represents the average ± SD ($n = 3$). P values were obtained using the Paired *t*-test of the GraphPad Prism Software (**$p < 0.01$).

in association with a sharp decrease of the proportion of S-phase cells.

Discussion

This study investigated the possible effects of mithramycin, known to be a strong inducer of fetal hemoglobin in erythroid cells from normal and β-thalassmic patients, on the mTOR pathway. The involvement of the mTOR pathway in erythroid differentiation is sustained by the finding that rapamycin, an inhibitor of mTOR activity is a potent inducer of erythroid differentiation of human leukemic K562 cells [18] and fetal hemoglobin production by β-thalassemic patients [19].

Of the major classes of mRNAs involved in the mTOR pathway (mTOR, rictor and raptor), the expression of raptor mRNA is strongly reduced by mithramycin treatment. The mechanism of action leading to this effect was studied *via* the promoter sequence of the raptor gene revealing several Sp1 binding sites. These transcription factors are highly expressed in K562 cells and expression does not change following MTH treatment. Its role in raptor gene expression is sustained by the finding that treatment of K562 cells with decoy molecules mimicking the Sp1-e site of the raptor gene promoter leads to raptor mRNA decrease (see Fig. 4). The hypothesis that one of the mechanisms of action of the G + C-selective DNA-binding drug mithramycin is the interaction with Sp1-like sequences causing inhibition of the binding of Sp1 with the raptor promoter in target cells is supported by the following results: (a) MTH strongly inhibits the interactions between Sp1 and Sp1-binding sites of the raptor promoter (EMSA assays); (b) MTH strongly reduces the recruitment of Sp1 transcription factor to the raptor promoter in intact K562 cells (ChIP experiments. A sharp decrease of p70S6 kinase phosphorylation and cell cycle alterations were reproducibly detectable in mithramycin treated K562 cells in agreement with the hypothesis that MTH inhibits, through raptor decrease, the mTORC1 activity.

This study does not allow us to conclude that inhibition of mTOC1 is required for erythroid differentiation, but simply that this issue deserves to be further investigated, as mTOR is expressed at high levels in erythrocytes [47]. On the other hand, the mTOR inhibitors rapamycin [18,19] and its analog everolimus [48] are strong inducers of erythroid differentiation of K562 cells and HbF induction in erythroid precursor cells of β-thalassemia patients. One possible explanation of this apparent discrepancy is that changes of mTOR activity can be associated to specific stages of erythroid differentiation, with significant differences between early and late/terminal stages of differentiation. Furthermore, the decrease of mTOR activity might be linked, instead to the overall erythroid phenotype, only to some erythroid features leading to high expression of embryo-fetal globin genes. In addition to changes at the transcriptional control level, translational control might also be involved both in activation of erythroid pathways, and the regulation of the expression of γ-globin genes [49,50]. This

specific issue was not addressed by the present study. However, hypoxia, a condition stimulating HbF in erythroid cells [51], is also associated with the decrease of mTORC1 activity [52] and alteration of the control of protein synthesis [53].

Whilst we cannot exclude additional mechanism(s) of action, the results reported here suggest that one of the biological effects of mithramycin might be the inhibition of Sp1 binding to the raptor gene promoter. In order to extend this observation to other erythroid cellular system, further research should be focused on erythroid precursor cells from normal subjects and β-thalassemia patients. Mithramycin has been proposed not only as inducer of HbF in thalassemic cells [26], but also as anti-tumor drug in cancer cells [54,55]. Therefore, our study might be of interest in the field of mechanism of action of anti-tumor compounds. Finally, the understanding of the mechanism of action of mithramycin might retain practical application in applied biomedicine. In fact, biochemical targets of MTH action (for instance raptor and Sp1) might be themselves novel biomolecular targets of therapeutic intervention based on more selective agents (such as antisense and shRNA molecules targeting mRNAs, or double stranded molecules targeting transcription factors). Interestingly, limiting this considerations to erythroid cells, the mTOR inhibitors rapamycin and everolimus are potent inducers of HbF in erythroid precursor cells from β-thalassemia patients [19,48] and RNAi-mediated Sp1 knockout induces high level of hemoglobinization in K562 cells [56].

Conclusions

This study presents evidence supporting the concept that alteration of raptor gene expression occurs during mithramycin-mediated induction of erythroid differentiation of K562 cells. One of the mechanisms of action of mithramycin is the inhibition of Sp1 binding to the raptor promoter, thereby strongly inhibiting the transcription of the raptor gene. In association with the MTH-mediated down-regualtion of raptor, mTORC1 associated functions such as p70S6 kinase phosporilation and cell cycle parameters were also altered.

Authors contributions

AF and RG participated in research design; NB, AF and JG conducted K562 cell culture; AF and GB performed EMSA experiments and conducted Sp1 decoy experiments; AF conducted Chromatin Immunoprecipitation experiments; MB performed Bio-plex experiments; AF, NB and EF conducted RT-PCR analyses; EF, GB and JG conducted FACS experiments; NB and AF performed Western blotting experiments; AF, NB and MB analyzed data; AF, RG and JG wrote or contributed to the writing of the manuscript.

Conflict of interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2014.11.005.

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