FEBS Letters 586 (2012) 3373–3378

journal homepage: www.FEBSLetters.org

Structural and functional characterization of the bacterial translocation inhibitor GE82832

Letizia Brandi [a,1], Sonia Maffioli [b,1], Stefano Donadio [b], Fabio Quaglia [a], Marco Sette [c], Pohl Milón [a,2], Claudio O. Gualerzi [a,*], Attilio Fabbretti [a,*]

[a] Laboratory of Genetics, Department of Biosciences & Biotechnology, University of Camerino, 62032 Camerino (MC), Italy
[b] NAICONS Scrl, Via Fantoli 16/15, 20138 Milano, Italy
[c] Department of Chemical Sciences and Technology, University of Rome-Tor Vergata, 00133 Roma, Italy

ARTICLE INFO

Article history:
Received 21 June 2012
Revised 10 July 2012
Accepted 11 July 2012
Available online 24 July 2012

Edited by Michael Ibba

Keywords:
Dityromycin
Translation
Translocation inhibitor
Natural product
Ribosome

ABSTRACT

The structure of GE82832, a translocation inhibitor produced by a soil microorganism, is shown to be highly related to that of dityromycin, a bicyclodecadepsipeptide antibiotic discovered long ago whose characterization had never been pursued beyond its structural elucidation. GE82832 and dityromycin were shown to interfere with both aminoacyl-tRNA and mRNA movement and with the Pi release occurring after ribosome- and EF-G-dependent GTP hydrolysis. These findings and the unusual ribosomal localization of GE82832/dityromycin near protein S13 suggest that the mechanism of inhibition entails an interference with the rotation of the 30S subunit "head" which accompanies the ribosome-unlocking step of translocation.

1. Introduction

In the last decades the disengagement of the pharmaceutical companies from antibiotic research and development has resulted in a dwindling of the antibiotic pipelines, just at the time when the need for effective antibiotics has dramatically increased by the emergence and spread of antibiotic-resistant "superbugs" which are killing hundreds of thousands of people each year [1,2]. To overcome the drastic shortage of new antibiotics, the world scientific community is called to make a renewed, focused, possibly combined effort to tackle the present "antibiotic emergency". After the initial hopes and the subsequent disappointments generated by combinatorial chemistry approaches to the generation of new anti-infectives, it seems clear that the most promising course to take is that of relying again on natural compounds, which were and remain the best source of antimicrobials [3–7]. The development of smart, high-content screening tests and the use of new generation libraries is expected to increase the chances of detecting bioactive natural compounds while the potentiality offered by modern developments in rational design, fragment based drug discovery and computational chemistry will be instrumental for the possible subsequent improvement of the identified molecules [8–14].

GE82832 is one of the few antibiotics recently identified by screening a library of secondary metabolites produced by soil microorganisms for bacterial translational inhibitors endowed with antibacterial activity [15]. GE82832 is produced by *Streptosporangium cinnabarinum* (strain GE82832) and consists of two bioactive isomeric substances (A and B), both having MW 1286 and containing an aromatic and a peptidic part. Functional tests have shown that GE82832 selectively inhibits exclusively bacterial protein synthesis in vitro and in vivo; furthermore, it was demonstrated that this molecule targets the small ribosomal subunit and inhibits the translocation step of elongation [15].

At the time of its identification, the structure of GE82832 had not been completely solved. Analysis of the physical and chemical properties of GE82832 suggested that this compound might be strictly related to the bicyclodecadepsipeptide dityromycin [16,17], a "neglected" antibiotic [18,19], discovered a long time ago but whose mechanism of action had never been determined and whose characterization had never been pursued beyond its antimicrobial spectrum and its structural elucidation [16,17].

In this study we have further characterized the mechanism of action and ribosomal localization of GE82832, and compared them

* Corresponding authors.
E-mail addresses: claudio.gualerzi@unicam.it (C.O. Gualerzi), attilio.fabbretti@unicam.it (A. Fabbretti).
[1] These authors have contributed equally to this work.
[2] Present address: Department of Physical Biochemistry, Max Planck Institute of Biophysical Chemistry, Goettingen, Germany.

0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.07.040

to those of dityromycin, demonstrating that these two compounds are structurally and functionally highly related.

2. Materials and methods

GE82832 was produced and purified by Biosearch Italia S.p.A. Gerenzano (VA), Italy; Dityromycin was a kind gift of Professor S. Ōmura (Tokyo).

ESI-MS data were recorded on an Ion Trap ESQUIRE 3000 Plus spectrometer (Bruker, Karlsruhe, Germany) equipped with an LC Agilent 1100 diode-array detector, using an Ascentis express Supelco RP18, 2.7 μm (50 × 4.6 mm) column kept at 40 °C, eluting at 1 ml min^{-1} with a 7-min linear gradient from 95:5 phase A [0.05% (v/v) trifluoroacetic acid in water]:phase B [0.05% (v/v) trifluoroacetic acid in acetonitrile] to 100% phase B.

2.1. Chemical reactivity tests

(a) Twenty microlitres of GE82832 or dityromycin solution (2 mg/ml in 20% DMSO) were added to AcOH:37% HCl 25:1 (v/v) (1 ml), (b) 20 μl of GE82832 or dityromycin solution were added to AcOH:37% HCl 1:1 (v/v) (1 ml) and (c) 20 μl of GE82832 or dityromycin solution were added to NaOH 1 M (1 ml). The solutions were left at room temperature for 10 min before LC–MS analysis.

2.2. Translation tests

In vitro mRNA translation, tests of individual translational steps, in situ probing of 16S rRNA cleavage by FeEDTA-generated hydroxyl radicals and the subsequent primer extension analysis were carried out as described [20–23].

2.3. Rapid kinetics analyses

MF-mRNA (or fluorescein-labeled MF-mRNA)-programmed pre-translocation complexes carrying deacylated $tRNA_{fMet}$ and fMetPhe-$tRNA_{Phe}$ in the P- and A-site, respectively, were prepared as described [24]. Fluorescence stopped-flow analyses were performed in 50 mM Tris–HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$ by rapidly mixing at 37 °C equal volumes (50 μl) of pre-translocation complex (0.2 μM in syringe 1) with or without antibiotic (20 μM) with EF-G·GTP (4 μM in syringe 2). Pi release from EF-G following GTP hydrolysis was monitored by the fluorescence change of MDCC-labeled phosphate-binding protein (PBP–MDCC) as described [24].

3. Results

When searching the proprietary ABL database [25], which contains properties of known microbial metabolites, we noticed that GE82832 showed a similar molecular mass and identical UV–vis spectrum to dityromycin, an antibiotic whose molecular structure had been elucidated over 30 years ago [16,17]. Although the genus *Streptosporangium* (the producer of GE82832) is distantly related to the genus *Streptomyces* (the producer of dityromycin), many precedents exist of representatives of the same chemical class produced by distant genera of *Actinobacteria* (e.g., glycopeptides) [26].

The correspondence between GE82832 and dityromycin was confirmed by the functional tests carried out in parallel with the two molecules. Indeed, GE82832 and dityromycin yielded essentially superimposable dose–response curves in the inhibition of mRNA translation and fMet-Phe-puromycin formation (Fig. 1). Furthermore, the topographical localization of GE82832 and dityromycin seems to be the same, as judged from the identical in situ hydroxyl radical cleavage pattern obtained in the presence of the

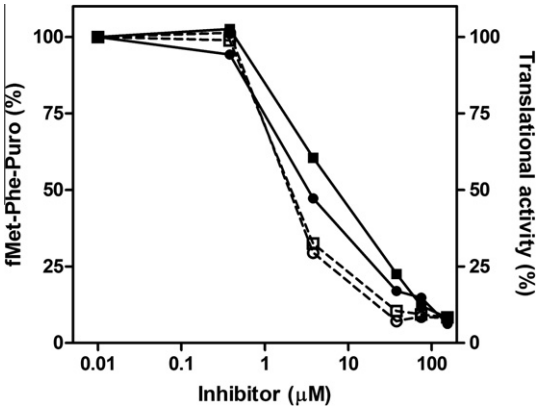

Fig. 1. Comparison of the inhibitory activities of GE82832 and dityromycin. Effect of increasing concentrations of GE82832 (●, ○) and dityromycin (■, □) on the EF-G-dependent translocation that allows formation of fMet-Phe-puromycin (●, ■) and on overall mRNA translation (○, □). Further experimental details can be found in materials ad methods.

two antibiotics (Fig 2A,B,C). These experiments show that both antibiotics affect the accessibility of the hydroxyl radicals in 16S rRNA helices h42 and h43 which are located in the head domain of the 30S subunit. Indeed, the accessibility of bases belonging to h42 located in proximity of ribosomal protein S13 is affected by the antibiotics with U1326 becoming more exposed to the cleavage while C1328 and A1329 are protected. In addition, also protected are the bases comprised between 1337 and 1340; these bases are located in a portion of the 30S forming part of the P-site gate and involved, together with the N-terminal portion of S13 and H38 of 23S rRNA, in the formation of inter-subunit bridge B1 [27]. Furthermore, the two antibiotics protect from cleavage bases belonging to h43. These are A1346, U1351, C1352 which are also located near S13 but in a more internal portion of the 30S head. These results not only are in agreement with the results of the DMS chemical probing which indicated that GE82832 protects A1324 and A1333 while exposing C1336 [15], but also demonstrate that the binding of the two antibiotics affects the structure of a broader region of the head of the 30S subunit, spanning from protein S19 to part of the gate forming the P-site and centered around protein S13, which represents a key element for aminoacyl-tRNA/mRNA translocation [28].

Thus, these results indicate that in addition to targeting with the same efficiency the same translational function, the two antibiotics have the same topographical localization and/or produce identical structural changes on the 30S ribosomal subunit.

On the basis of these results the two molecules were subjected to further comparisons. Due to the limited availability of both GE82832 and dityromycin, comparisons were based on LC–MS analyses of the intact compounds and after parallel chemical reactions. LC analyses (see Material and Methods) confirmed that both dityromycin and GE82832 elute as complexes of two isomeric substances with similar retention times (4.6 and 4.4 min, respectively), thus showing comparable lipophilicity. Furthermore, both dityromycin congeners showed UV spectra (with maxima at 280 and 310 nm), consistent with literature data [16,17] and superimposable on those measured for both GE82832 congeners (data not shown). MS analysis of dityromycin in positive ionization mode presented a monocharged ion at *m/z* 1311, corresponding to the sodiated molecule $[M+Na]^+$ while negative ionization mode led to an ion at *m/z* 1401, corresponding to the trifluoroacetic acid adduct. These data are consistent with the mass of 1288 amu reported by Ōmura's laboratory [16,17]. Under the same ionization conditions, GE82832 showed monocharged ions of *m/z* 1309 and

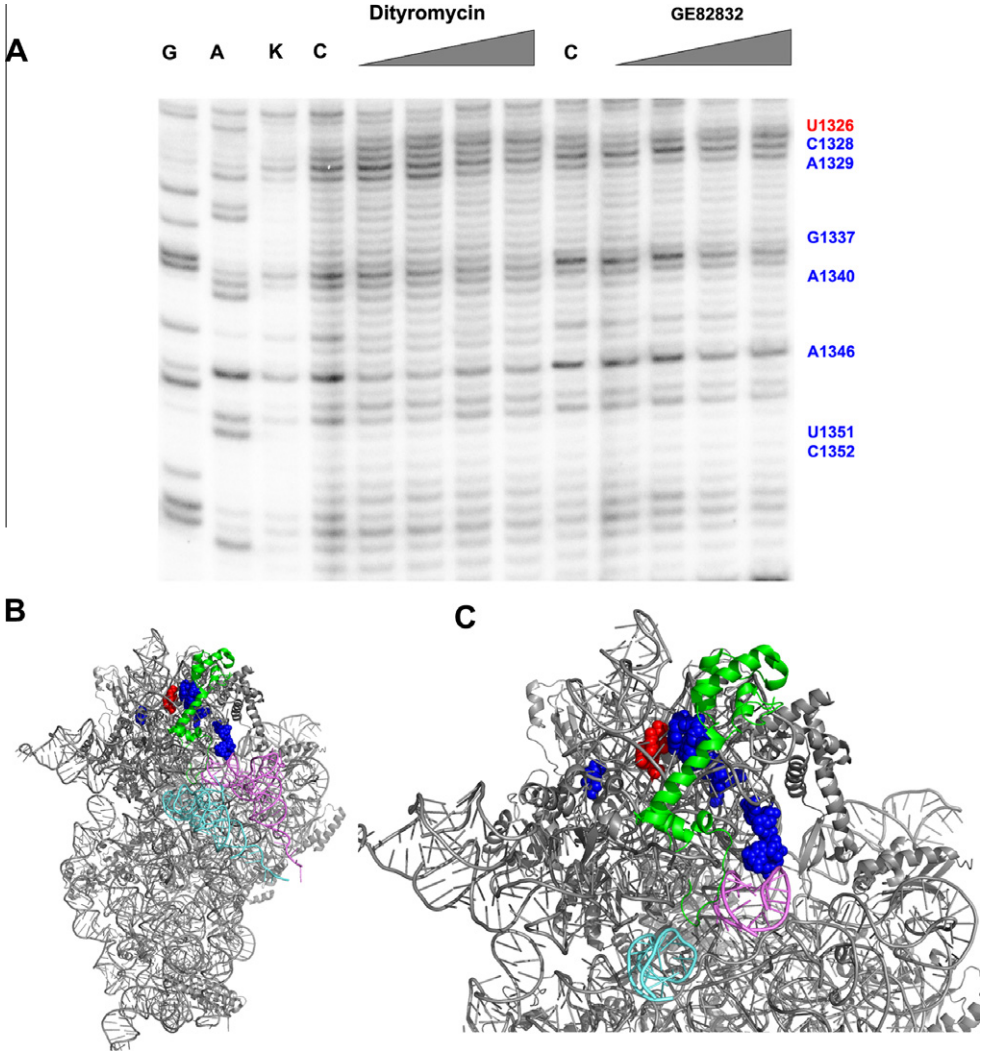

Fig. 2. Comparison of the in situ accessibility of 16S rRNA in the presence of GE82832 and dityromycin The topographical localization of GE82832/dityromycin on the 30S ribosomal subunit was determined from the primer extension analysis of the cleavage sites generated on the 16S rRNA in situ by hydroxyl radicals in the presence of increasing concentrations of GE82832 (left) and dityromycin (right). The bases protected and more exposed in the presence of GE82832 and dityromycin are indicated at the right-hand side of the gels in blue and red script, respectively. The lanes G, A, K, C correspond to sequencing lanes (G, A) and to the 16S rRNA not subjected to cleavage (K) or subjected to cleavage in the absence of antibiotics (C). (B) The topographical localization of ribosomal protein S13 (green) and of the sites whose accessibility is increased (red) or decreased (blue) in the presence of GE82832/dityromycin are indicated within the 3D structure of the 30S ribosomal subunit (PDB file 1YL4); (C) close-up view of the localization of S13 and of the 30S ribosomal subunit sites protected and more exposed in the presence of GE82832/dityromycin. The color code is the same as in panel (B). Further details can be found in Ref. [23].

1399, corresponding to $[M+Na]^+$ and TFA adducts, respectively, consistent with a 2-amu difference between GE82832 and dityromycin. A close structural relationship between the two molecules is further strengthened by MS/MS experiments, which yielded the same fragmentation pattern for GE82832 and dityromycin (Fig. 3A,B). Both molecules were then reacted with AcOH: 37% HCl 25:1 (v/v) at room temperature. After 10 min, LC–MS revealed the disappearance of the starting compounds and the formation of lipophilic derivatives in a 1:1 ratio, having $[M+Na]^+$ at *m/z* 1347 and 1383 for dityromycin, and at 1345 and 1381 for GE82832 (data not shown). The 36-amu differences and the isotopic patterns of the mass ions suggested that these products were formed by addition of one (*m/z* 1347 and 1345) or two molecules (*m/z* 1383 and 1381) of hydrochloric acid to both dityromycin and GE82832, consistent with the presence of the epoxy-hydroxy-dehydro-isoleucine moiety. Under more drastic conditions, (AcOH:37% HCl 1:1 v/v) smaller fragments, lacking chlorine atoms and having *m/z* 1148 and 1146, were observed for dityromycin and GE82832, respectively, probably due to the loss of the halogenated residue followed by a dehydration. A preliminary NMR spectrum, obtained on a partially purified sample of GE82832, highlighted the presence of signals belonging to a di-tyrosine moiety in a peptide structure containing also signals assignable to *N*-methyl valine, *N*,*N*-dimethyl-threonine and phenylglycine, residues that are also found in dityromycin (Fig. S1 and Table S1).

All these data strongly suggest that the structure of GE82832 is identical to that of dityromycin (Fig. 3C), but for the presence of minor chemical differences. The 2-amu difference might result from an additional unsaturation present somewhere in GE82832. However, alkaline treatment of the two molecules (1 M NaOH) showed the formation of smaller fragments having *m/z* 753 and 737 for dityromycin and GE82832, respectively, suggesting that the 2-amu difference may originate from a combination of at least two modifications in different parts of the molecule. To discriminate among the various possibilities, further investigations are necessary on larger amounts of purified compounds.

The following experiments were carried out to obtain a deeper understanding of the mechanism by which translocation is

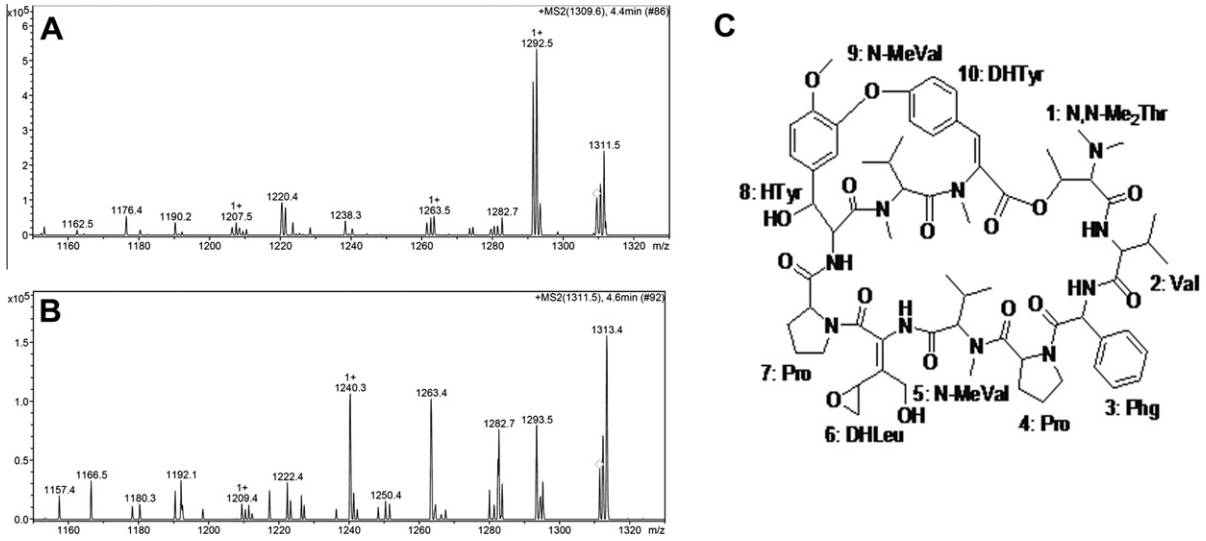

Fig. 3. Comparison of the MS spectra obtained with GE82832 and with dityromycin and chemical structure of dityromycin (A) GE82832; (B) dityromycin; (C) chemical structure of dityromycin (from [17]). Further details can be found in the text.

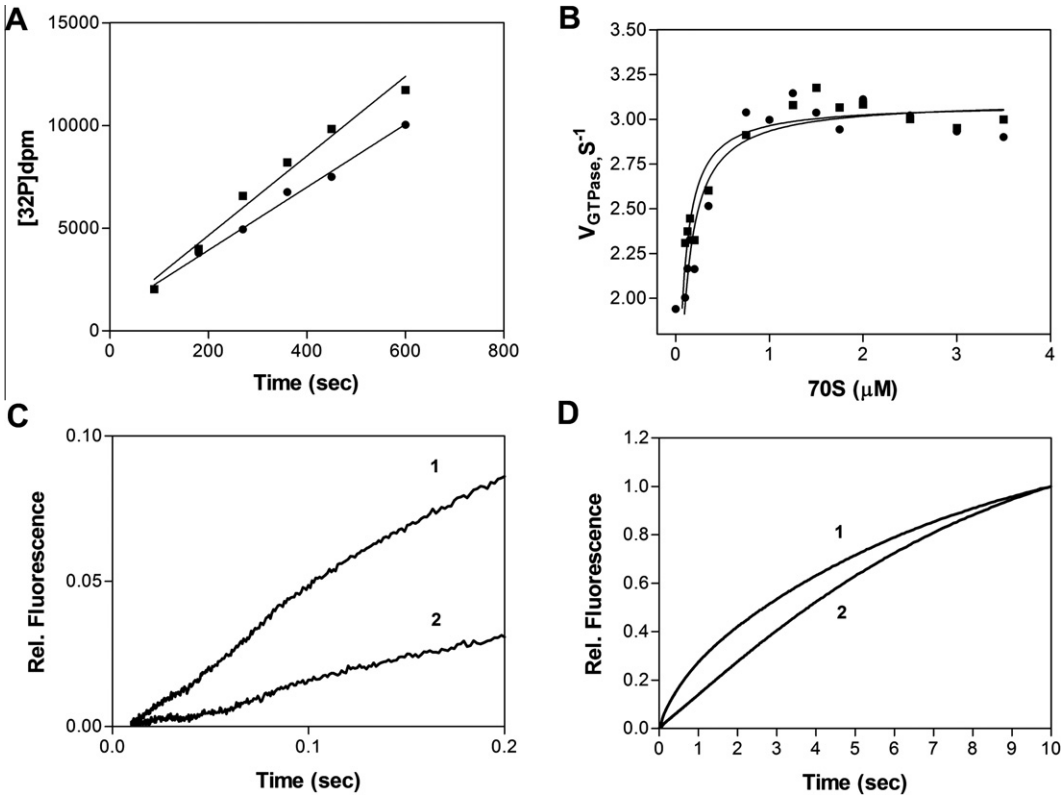

Fig. 4. Effect of GE82832/dityromycin on the ribosome and EF-G-dependent GTP hydrolysis and Pi release. (A) Multiple turnover EF-G-dependent GTP hydrolysis; (B) single round EF-G-dependent GTP hydrolysis in the presence (●) or absence (■) of 20 μM of GE82832/dityromycin; (C and D) Pi release from EF-G-GDP-Pi on a shorter (C) and longer (D) time-scale. Tracings marked "1" and "2" represent the kinetics recorded in the absence and presence of 20 μM GE82832/dityromycin, respectively. Further experimental details can be found in materials ad methods.

inhibited by GE82832/dityromycin. The identical results obtained with both GE82832 and dityromycin indicate that the antibiotics do not inhibit the ribosome- and EF-G-dependent hydrolysis of GTP, either when this activity is measured in a multiple turnover mode (Fig. 4A) or as single round reaction (Fig. 4B). These data indicate that the ribosomal binding and affinity of EF-G for the ribosomes and the activation of the GTPase catalytic center of the factor are not affected by the antibiotics. On the other hand, the release of Pi from EF-G (Fig. 4C,D) as well as the movement of both mRNA (Fig. 5A) and aminoacyl-tRNA (Fig. 5B) are substantially slowed down in the presence of the antibiotics, suggesting that the inhibition occurs at the level of ribosome "unlocking" which precedes these three activities (Fig. 5C).

Finally, the reported antibacterial spectrum of dityromycin (active against *Bacillus*, *Corynebacterium* and *Clostridium*, but inactive against *Staphylococcus aureus* and Gram-negative bacteria [17] is

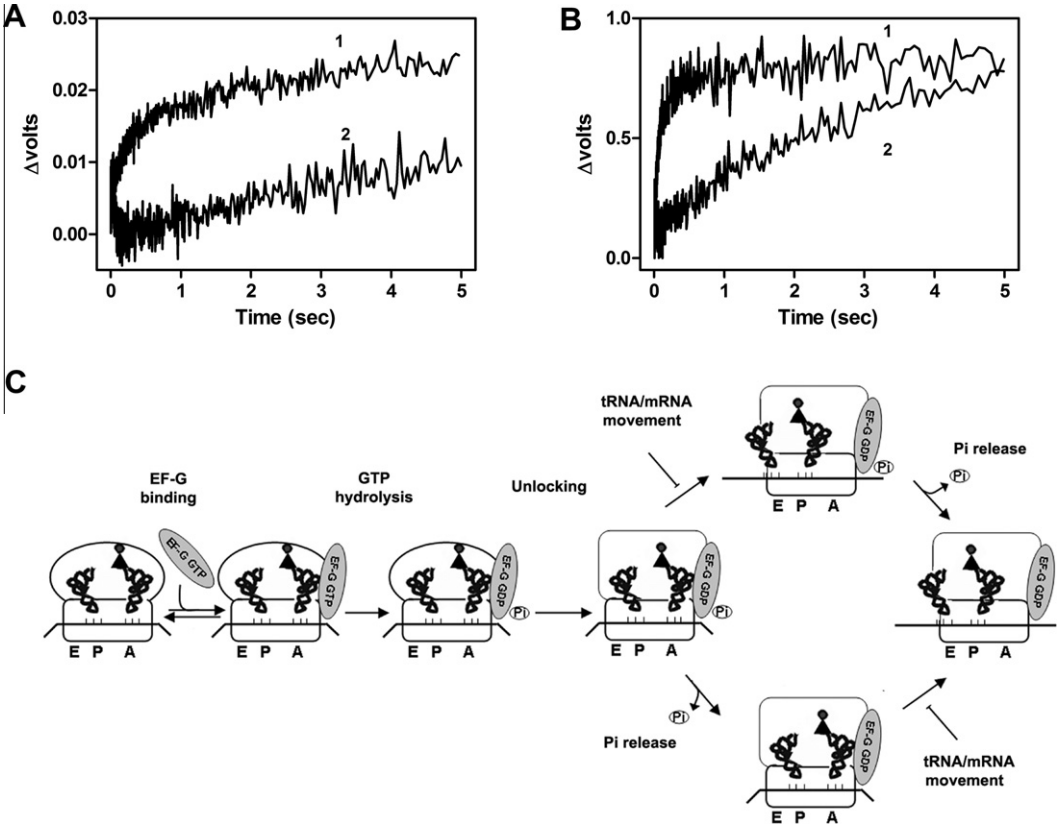

Fig. 5. Effect of GE82832/dityromycin on the EF-G-dependent tRNA/mRNA translocation Effect of GE82832/dityromycin on the kinetics of EF-G-dependent (A) mRNA; (B) aminoacyl-tRNA translocation. Tracings marked "1" and "2" represent the kinetics recorded in the absence and presence of 20 μM GE82832/dityromycin, respectively. Further experimental details can be found in Materials ad Methods; (C) scheme of the various steps involved in translocation.

consistent with that observed for GE82832 [15]; this is an additional indication that GE82832 and dityromycin have essentially the same structure.

4. Discussion

This study allows us to attribute simultaneously structural information to an inhibitor for which only the target had been identified and a specific function to an antibiotic for which nothing but the structure was known. In fact, here we have demonstrated that the chemical structure of GE82832, the translocation inhibitor identified by HTS of a natural library of secondary metabolites of soil microorganisms [15], corresponds almost completely to that of dityromycin, an antibiotic whose structure had been elucidated but whose mechanism of action had never been investigated [16,17]. The minor difference (two units of mass) detected between GE82832 and dityromycin is irrelevant for the biological activity of these two molecules, which displayed identical properties and activities in all tests performed. In addition, also the microbiological spectrum of action of GE82832, as determined by the preliminary tests, proved to be very similar to that reported for dityromycin [16]. It should be mentioned that cross-linked Tyr residues similar to that present in GE82832 and dityromycin, besides being the hallmark of glycopeptides [26], are also present in plant metabolites such as bouvardin and related compounds that inhibit protein synthesis in eukaryotes [29].

Furthermore, our data shed light on the mechanism by which bacterial translocation is inhibited by GE82832/dityromycin. Translocation proceeds in steps beginning with a pre-translocation complex (Fig. 5C). Overall, the process is driven, both kinetically and thermodynamically, by elongation factor G (EF-G) whose five-domains change conformation upon GTP hydrolysis and release of inorganic phosphate (Pi), in a process coupled to conformational changes of the ribosome that facilitate the movement of both aminoacyl-tRNA and mRNA to yield a post-translocation complex (Fig. 5C).

Analysis of the results of the various translocation steps obtained in the presence of GE82832/dityromycin indicates that these antibiotics do not inhibit the formation of the pre-translocation complex in which the aminoacyl-tRNAs assume hybrid binding positions [30], the binding of EF-G-GTP [31], the rotation of the 30S subunit relative to the 50S subunit and the swiveling motion of the head of the 30S subunit [32–34] and the activation of the EF-G-dependent GTP hydrolysis. On the other hand, GE82832/dityromycin likely interferes with the subsequent step of translocation, namely the rearrangement or "unlocking" of the ribosome which allows the movement of aminoacyl-tRNA and mRNA and the release of Pi from EF-G-GDP [35]. Although these steps are not strictly linked with one-another and each step can be inhibited independently [35–37], none of the antibiotics tested (paromomycin, spectinomycin, hygromycin B, tetracycline, streptomycin) was able to inhibit both steps [37]. Thus, the mechanism of translocation inhibition by GE82832/dityromycin is special insofar as this antibiotic inhibits the movement of both aminoacyl-tRNA and mRNA and decreases the rate of Pi release.

In light of these findings we can surmise that GE82832/dityromycin interferes with ribosome-unlocking, a step which entails an increased mobility of the head of the 30S subunit and is necessary for Pi release and aminoacyl-tRNA/mRNA movement (Fig. 5C). The effects of GE82832/dityromycin on the accessibility of 16S rRNA to hydroxyl radical cleavage (Fig. 2) and chemical modifications [15] near protein S13, in the head region of the subunit, are

fully compatible with the hypothesis that GE82832/dityromycin might interfere with the internal mobility of the 30S subunit, which is rate-limiting for translocation [35] rather than with the ratcheting mechanism of the two subunits as previously suggested [15].

Acknowledgments

We are grateful to Professor S. Omura (Tokyo) for providing a sample of dityromycin and to Professor Cynthia L. Pon (Camerino) for her invaluable discussions and suggestions in the preparation of this manuscript. This work was started with the financial support of the EC Grant (Contract QLRT-2001-00892 "Ribosome inhibitors") to C.O.G. and L.B. and later partially supported by a Grant from Regione Lombardia (Bando ATP). We also acknowledge the early contribution of former colleagues at Biosearch Italia, spa (Gerenzano).

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.febslet.2012.07.040.

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