The G11778A LHON mutation does not enhance ethambutol cytotoxicity in a cybrid model

R. Pommer¹, S. Schoeler¹, C. Mawrin², R. Szibor³ and E. Kirches¹

Institutes of ¹Neuropathology and ³Forensic Medicine, Otto von Guericke University Magdeburg, ²Institute of Neuropathology, Friedrich Schiller University Jena, Germany

Key words
LHON – ethambutol – cybrid – ATP – mtDNA

Abstract. Leber’s hereditary optic neuropathy (LHON) is a maternally inherited mitochondrial disorder, leading to a selective loss of retinal ganglion cells (RGC) and degeneration of the optic nerve, which results in severe visual impairment or even blindness. The primary causes are point mutations of the mitochondrial DNA (mtDNA), associated with amino acid exchanges in complex I of the electron transport chain (ETC), which are thought to disturb oxidative ATP generation in the mitochondria. The major side effect of the antibiotic ethambutol, commonly used in tuberculosis therapy, is a retinopathy, which may lead to selective RGC loss, if not detected in an early stage. Moreover, LHON was reported to be elicited by ethambutol in some mutation carriers. Objective: The present study intended to measure a possible synergism between mitochondrial dysfunction, caused by the most common LHON mutation (G11778A) and caused by ethambutol, which may lead to a higher cytotoxicity of the drug in LHON cells. Material: An NT2/D1 teratoma-derived LHON cybrid line and the parental cells. Method: Determination of ethambutol toxicity in both lines, using a microtiter tetrazolium assay, luminometric measurement of ATP/ADP ratios and determination of mtDNA copy numbers by Real-time PCR. Results: Short-term ethambutol toxicity occurred only at submicromolar concentrations, far beyond the estimated plasma peak concentrations of patients under antibiotic therapy. No significant difference occurred between both cell lines. The ATP/ADP ratios in the cybrids were surprisingly low, but showed no correlation with the mutational status of drug-treated cells. The mtDNA copy number of treated LHON and parental cells did not differ significantly. Conclusions: Ethambutol shows no synergism with the most common primary LHON mutation with respect to mitochondrial energy production or mtDNA replication in cybrid cells, although the issue of ATP decline should be further addressed in neuronally differentiated cybrids with complete OXPHOS dependency.

Introduction

Leber’s hereditary optic neuropathy (LHON), a neuroophthalmologic disease originally discovered by the German ophthalmologists von Graefe and Leber in the middle of the 19th century [Leber 1871, von Graefe 1858], is meanwhile known to be a maternally inherited mitochondrial disorder [Wallace et al. 1988]. Its primary cause are point mutations of the mitochondrial DNA (mtDNA), which lead to the exchange of a single amino acid of NADH : ubiquinone oxidoreductase, a large L-shaped protein complex (complex I) in the inner mitochondrial membrane, donated to introduce NADH derived electrons into the electron transport chain (ETC). The location of the mutations in complex I and early biochemical studies suggested an altered ubiquinol site and enhanced product inhibition of the mutated complex [Carelli et al. 1999, Majander et al. 1996]. While a decreased enzyme activity in mutant lymphoblasts, skin fibroblasts or cybrid cells remains controversial for the most common mutation G11778A, complex I respiration was found to be decreased in several studies [Brown et al. 2000, Floreani et al. 2005]. Moreover, the neurologist Valerio Carelli and coworkers demonstrated a clear defect in oxidative ATP synthesis [Baracca et al. 2005] as well as in cellular ATP content under metabolic stress conditions in a cybrid model [Zanna et al. 2003, 2005], resulting in enhanced apoptotic sensitivity of the LHON cybrids. These and other
observations favored strongly the hypothesis of retinal ganglion cell (RGC) death, mediated by a combination of neuronal ATP deficiency and enhanced superoxide generation in complex I [Carelli et al. 2004a]. According to this hypothesis, the selective vulnerability of RGC may be caused by their high oxidative energy demand. The axon sections in the retinal nerve fiber layer are unmyelinated, thus requiring more mitochondrial ATP to save the plasma membrane potential. This argument is underlined by the stronger histochemical cytochrome c oxidase staining of these intracellular portions of the optical axons [Carelli et al. 2004b]. Although the primary causes of LHON are known, additional factors influence the likelihood of disease in mutation carriers. This can be concluded from the incomplete penetrance, ranging between 25% and 80% in different populations and studies [Mackey and Buttery 1992, Man et al. 2003, www.ncbi.nlm.nih.gov/omim], and from the strong male predominance. While the latter suggests the participation of a yet unknown, recessive X-allele [Hudson et al. 2005], the disease-promoting role of alcohol abuse [Sadun et al. 2002, 2003, 2004] underlines the importance of epigenetic factors, targeting the retina.

One such factor may be the antibiotic ethambutol (EMB), which has commonly been used since 1961 to treat tuberculosis [Fraunfelder et al. 2006]. The most prominent EMB side effect is a retinopathy, which resembles LHON in multiple features and was called ethambutol optical neuropathy (EBON) by Ikeda and colleagues [Ikeda et al. 2006]. This type of retinopathy is also characterized by a severe loss of visual acuity, disturbed color vision and central scotoma. Like LHON, the disease predominantly affects the smaller RGC of the macula and the corresponding smaller axons of the papillomacular bundle. In contrast to LHON, the disease is most often reversible after changing the antibiotic regime, if detected at an early stage by the ophthalmologist. It may, however, proceed to severe visual impairment or blindness, in some cases even after early detection of the symptoms and drug omission. In recent years, some cases of G11778A mutation carriers have been described, which developed LHON during antibiotic EMB treatment and were originally recognized in this way as mutation carriers (summarized by [Ikeda et al. 2006]).

This situation led us to examine the cytotoxicity of EMB in a cybrid cell culture model. For this purpose, NT2/D1 teratoma-derived transmitochondrial cybrid cells, in which the tumor mtDNA had been completely replaced by the mtDNA of a G11778A LHON patient [Wong et al. 2002], were compared with the parental cell line. This offers the possibility to compare cells with and without the LHON mutation under conditions of an identical chromosomal background. After establishing EMB dose response curves in a cytotoxicity assay, ATP/ADP ratios were measured as a direct indicator of altered oxidative phosphorylation (OXPHOS). Furthermore mtDNA copy numbers were measured with respect to the hypothesis, that EMB may inhibit mtDNA replication and, thus, indirectly impair OXPHOS.

Materials and methods

Cell lines and genetic confirmation

The Ntera-2 clone D1 (NT2/D1) teratoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The NT2/D1-derived LHON cybrid clone was a kind gift from Prof. Dr. Gino Cortopassi and Prof. Dr. Alice Wong from the University of California (Davis, CA, USA). The nuclear genetic identity of both cell lines was proven by identical allelic patterns of a collection of microsatellite markers and amelogenin, using the MenType Nonaplex II PCR kit (BioType, Dresden, Germany) as described elsewhere [Schoeler et al. 2007]. In brief, the polymorphic marker regions were PCR-amplified with fluorescence-labeled primers and reaction products underwent electrophoresis on an ABI310C capillary sequencer (Applied Biosystems, Foster City, CA, USA), followed by automatic allele identification by the GeneScan software (Applied Biosystems). No allelic differences were observed. The presence of 100% mutant G-11778A mtDNA in the LHON cybrid clone was proven by direct sequencing and a specific PCR-RFLP assay, as described in detail elsewhere [Schoeler et al. 2007]. In brief, the
latter assay consisted of a PCR of the ND4 region of the mtDNA, followed by a digest with the restriction endonuclease SfaNI. While the mutation confers resistance to the digest, the wild type is cut. 6-FAM-labeled PCR fragments were separated on an ABI310C capillary sequencer, followed by automatic fragment identification by the GeneScan software. The stability of the LHON mutation was checked over the whole time period of the work. No remaining wild type DNA could be measured within the sensitivity limit of the assay (< 1%) in all subsequent rounds of cell culture. The absence of the three primary LHON mutations at nucleotide positions 14484, 3460 and 11778 in the NT2/D1 line was checked by direct sequencing and for the latter two mutations with an PCR-RFLP assay, as described elsewhere [Schoeler et al. 2007]. Furthermore, sequencing revealed no other pathogenic mutations which distinguished the mtDNAs of the two clones. The only additional differences observed were known polymorphisms.

**Cell culture and EMB treatment**

All cell lines were cultured in high glucose (4.5 g/l) DMEM with a lowered bicarbonate content (1.5 g/l) from ATCC, supplemented with 1 mM sodium pyruvate, 4 mM L-glutamine, 10% fetal calf serum (EUGold, PAA, Linz, Austria) and 1% penicillin/streptomycin (PAA). Cells were cultured in T75 flasks at 37 °C in 5% CO₂ atmosphere, until 80% confluency was reached. They were split by trypsinization in a ratio of 1:4 or higher. For experiments in 96-, 48- or 24-well plates, an adequate cell number per well (see results section) was seeded and incubated for 24 h. After this preincubation, various concentrations of EMB (Riemer Arzneimittel AG, Greifswald, Germany) were applied for the indicated time periods, followed by microtiter-tetrazolium (MTT) cell survival assay. For ATP/ADP and mtDNA copy number assays, the cells were seeded in the indicated density (see results) in 6-well plates, treated with various EMB concentrations and subjected to lysis in ATP buffer or DNA extraction, respectively. Since the differentiation status of the cybrid lines was suspected to influence their OXPHOS dependency, cell survival and ATP/ADP ratios were also measured, following a short-term differentiation with retinoic acid (RA), which stopped cell proliferation. This was achieved by seeding cells as usual, but replacing DMEM after a 24-h incubation by serum-free Neurobasal (Invitrogen, Karlsruhe, Germany), enriched with 5% B27 supplement (Invitrogen) and 10 μM all-trans retinoic acid (RA) from Sigma (St. Louis, USA). Cells were incubated in this medium for 4 days, prior to the indicated ethambutol treatment.

**MTT assay**

For the microtiter-tetrazolium assay one volume of cell culture medium, containing 1.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterrazolium (MTT, Sigma, St. Louis, USA) was added to the culture wells and incubated at 37 °C for further 2 h. Thereafter, the medium was replaced by DMSO, which lysed the cells and dissolved the generated formazan dye. Optical densities (OD) at 562 nm (reference wavelength 630 nm) were measured in a microplate reader, and cell survival was expressed as the OD percentage, normalized to untreated controls.

**ATP/ADP ratio**

After trypsinization, cells were shortly washed 2 times in phosphate-buffered saline (PBS), resuspended in 300 μl boiling ATP buffer (100 mM Tris pH 7.75, 10 mM KCl, 4 mM MgCl₂) and boiled for further 2 min. After a short high-speed centrifugation, the supernatant was distributed into two fresh tubes. After cooling to room temperature, 1.5 mM phosphoenolpyruvate (Sigma) and 6 units/ml pyruvate kinase (Sigma) were added to 1 of the 2 aliquots and incubated for 30 min at room temperature, to phosphorylate all ADP in the sample to ATP. After this step, the aliquot was boiled for 1 min, to destroy the pyruvate kinase activity. Then 50 μl of an appropriate dilution of both lysates (1:20 – 1:50) was mixed with 50 μl of luciferase reaction mix (CLS II ATP kit, Roche, Mannheim, Germany). The resulting luminescence was immediately measured in a TD20/20 luminometer (Turner Design Instruments, Sunnyvale,
spss under EMB treatment with the untreated

ATP/ADP ratios and mtDNA copy numbers

Statistical analysis

Comparisons of surviving cell fractions, ATP/ADP ratios and mtDNA copy numbers under EMB treatment with the untreated controls were performed using a 1-way ANOVA, to elucidate whether the drug generally had an effect on these parameters. The specific drug concentrations, which exhibited a statistically significant difference to the controls, were identified by a Dunnett post-hoc test. Significant differences between the two cell lines for a given EMB concentration were determined by a t-test. All calculations were performed with the software package SPSS 13 (SPSS, Chicago, USA), assuming significance for p < 0.05. All diagrams show means ± SEM.

Results

Cell survival under EMB treatment

30,000 cells were seeded in 24-well plates in DMEM, incubated for 24 h and treated for another 24 h with the EMB concentrations indicated in Figure 1. The toxic effects, which could be measured in the MTT cell survival assay, were surprisingly low, even if umolar concentrations of the drug were applied for this short time period. Less than 30% of cells died at 2.7 mM, while the relevant concentrations in vivo are thought to be in the range between 20 and 80 μM (see Discussion). However, toxicity became significant (p < 0.01) in both cell lines for concentrations above 1.2 mM in the Dunnett test and was clearly dose-dependent. Although the dose response curve for the LHON mutant was beneath that one of the wild type over the whole concentration range, the difference between the lines only became significant (p < 0.01) for the highest concentration tested (2.7 mM), which is thought to be irrelevant in vivo.

To elucidate possible effects of the plate type used, of cybrid differentiation and of EMB exposure times, several similar experiments were performed. They all had essentially the same results, and are, therefore, not shown in detail in separate Figures. All experiments revealed a clear dosage-dependent toxicity of the drug, but no proof of a higher sensitivity of the LHON mutant as compared to the wild type.

In one series of experiments, 3,000 cells per well were seeded into 96-well plates and the same EMB concentrations, as shown in Figure 1, were applied for 24 h. The toxicity
of the drug seemed to be somewhat higher in this assay, since the maximal fraction of dead cells reached about 40% for 2.7 mM EMB. The slight difference between both plate types may be due to the higher percentage of cells, which are lost during sucking the cell culture liquid out of the wells, since EMB seemed to reduce the adhesion of the cells to plastic surfaces. The draining of cells with lowered adhesion becomes more prominent in smaller wells. In this assay variant, the dose response curve of wild type NT2/D1 cells was usually below that of one of the LHON mutant, although this difference again became significant only for the highest concentrations above 2.4 mM.

Even short-term differentiation with retinoic acid (RA) did not lead to an essentially different outcome. For this purpose, 5,000 cells were seeded into 48-well plates, followed by 4 days RA differentiation, prior to the 24 h EMB treatment in the same concentration range, as shown in Figure 1. Again, a clearly dosage dependent toxicity of the drug occurred, which was very similar to that of undifferentiated cells. No elevated sensitivity of the mutant clone was found.

In vivo the drug influences the retinal ganglion cells (RGC) over long-time periods of usually several months. Although a chronic treatment could not be simulated in the MTT assay, the time period of EMB incubation of the undifferentiated cells was extended in one experimental series to the possible maximum of 4 days. This limit resulted from the growth of the untreated controls which died about 24 h after reaching confluency. In all, 2,000 cells were seeded in 96-well plates. After 24 h of preincubation in DMEM, they were treated with the established EMB concentrations for a time period of 4 days, prior to MTT assay. After this prolonged incubation, toxicity reached a maximum around 45% cell death for 2.7 mM EMB, very similar to the results obtained for the 24-h exposure. Again, the dosage-dependent effect was statistically significant, without increased response of the mutant.

Due to the limited EMB toxicity in the MTT assay, we intended to find out whether extremely high concentrations of the drug may cause a more pronounced cell death in the LHON cybrids. Extending the concentration range to 12 mM lead to death of more than 80% of the cells, without elevated sensitivity of the mutant clone. While 50% death had not been reached in the previous experiments, the LD$_{50}$ could now be determined to be around 4 mM in both cell lines. Taken together, all types of MTT assays did not reveal a higher sensitivity of the mutant against the cytotoxic effect of EMB.

For concentrations above 0.5 mM, a clear morphologic change occurred in both lines. Small vacuoles appeared in phase-contrast micrographs. Number and mean diameter of these vacuoles increased with EMB concentration, but no obvious difference was visible in the microscope between both cell lines. After 24 h in 2.7 mM of the drug, the cytoplasm was filled with large numbers of these vacuoles (Figure 2). Their origin and impact for the cells are yet unknown.

**ATP/ADP ratio**

Here 100,000 cells per well were seeded into 6-well plates, incubated 24 h in DMEM and exposed to 0 (control), 0.6 or 2.7 mM EMB for 24 h, prior to the luminometric ATP/ADP assay. For experiments with differentiated cybrids, only 50,000 cells were initially plated, but a 4-day differentiation was
included in the protocol, prior to EMB treatment.

Generally, the energetic status of the cybrids was surprisingly low. The untreated LHON mutants in DMEM did not show per se a significantly lower ATP/ADP ratio, when compared with untreated NT2/D1 cells. This would have been in concordance with a hypothesis of the literature, assuming that proliferating cybrid cells in DMEM posses a largely nonoxidative (glycolytic) energy metabolism, which masks the effects of the mutations. However, the LHON cells even exhibited a higher ratio, when compared with the wild type ($p < 0.05$), a rather unexpected result (Figure 3). EMB selectively decreased the ratio in the mutant ($p < 0.05$), thus, leading to identical ATP/ADP ratios in mutant and wild type. This experiment was not finally conclusive. It suggested no general impact of EMB on OXPHOS, since the energy status of the wild type did not worsen under the influence of the drug. On the other hand, a possible influence selectively in the mutant clone was

Figure 2. Phase contrast micrograph showing relatively normal morphology, but accumulation of large amounts of vesicles in cyrid cells with the G11778A LHON mutation, as well as in parental cells, incubated with 2.7 mM EMB. Untreated cells are shown in A (NT2/D1) and B (LHON cybrids), while micrographs C (NT2/D1) and D, E (LHON cybrids) show cells after EMB exposure. The bars represent 50 μm.
inconclusive, since the statistically significant effect of EMB in this clone depended completely on the aberrant behavior of the untreated LHON cells (Figure 3).

For this reason and due to the suggested glycolytic energy metabolism of the undifferentiated cybrids in DMEM, the experiment was repeated with differentiated cells (Figure 4), which were thought to exhibit enhanced OXPHOS dependency. In this case, no significant differences at all occurred, neither between the cell lines, nor between treated and untreated groups. Taken together, these experiments suggest no major influence of EMB on OXPHOS.

**mtDNA copy number**

150,000 cells were seeded into each well of a 6-well plate. After 24 h in DMEM, 0 (control), 0.6 or 2.7 mM EMB were added. The cells were incubated in this medium for 5 days, to allow some mtDNA turnover. The copy number of mtDNA, normalized to a nuclear single copy gene (p53), was assayed by SYBR green Realtime PCR in the extracted DNA samples (Figure 5). The cells contained a mean of about 600 mtDNA copies per p53 copy. Although, the mtDNA content in the parental cell line seemed to be slightly higher as compared with the LHON cells, this difference did not reach statistical significance. EMB had no influence on the mtDNA content, neither in the wild type, nor in the LHON cells, suggesting no influence on mtDNA replication.

**Discussion**

In a series of 24 patients with EMB-induced retinopathy, Hwang and colleagues [Hwang et al. 2003] did not find any of the known primary LHON mutations. This suggested that the mutation incidence in such patients is not increased. However, this result does not conclusively exclude a synergism between genetic status and drug, since mutation carriers are relatively rare in the general population. On the other hand, several carriers of the G11778A transition had been identified by the development of LHON during tuberculosis therapy with EMB, suggesting that the drug favors disease outbreak. This suggestion was underlined by four cases (reviewed by [Ikeda et al. 2006]), in which the age of LHON onset in two males and two females was between 53 and 70 years, while sporadic or familial LHON disease usually affects young men in their first three decades of life.

While the neurotoxic side effects of EMB are well-documented in tuberculosis patients, the toxicity has not been well-defined in RGC cultures. In the last years, two groups investigated these toxic effects in dissociated rat reti-
The response curves suggested an sensitivity to EMB treatment was not surprised compared to primary neuron cultures, the lower neurotoxic and are known to be less sensitive against various teratoma cybrids, used in the present study, cur at much lower concentrations. Since the apoptotic effects can not determine the long term proapoptotic effects of the drug, which might occur at much lower concentrations. Since the teratoma cybrids, used in the present study, are known to be less sensitive against various neurotoxic and proapoptotic stimuli as compared to primary neuron cultures, the lower sensitivity to EMB treatment was not surprising. The LD_{50} was around 4 mM for a 24-h application. A selectively higher sensitivity of the LHON cybrids was not observed. Interestingly, at drug concentrations of 500 μM or higher, many vacuoles accumulated in the cytoplasm. With respect to this feature, the cybrids behaved largely like cultured RGC, which accumulated these vacuoles at a concentration range between 700 and 1,000 μM [Yoon et al. 2000]. The nature of these vacuoles remains unknown. They seemed to be surrounded by only a single membrane and, thus, not to be of mitochondrial, but eventually of ER or Golgi origin. Although the cytoplasm may become densely filled with vacuoles, their appearance is reversible, since they disappeared after removing EMB from RGC cultures.

The mechanism of EMB neurotoxicity remains unclear up to now, but a direct attack of OXPHOS complexes had been hypothesized as one possibility. The known copper chelating effect of the drug was suggested to lead to inhibition of cytochrome c oxidase (COX) activity [Ikeda et al. 2006]. This inhibition may cause a significant reduction of electron flux through the ETC, thus, diminishing oxidative ATP synthesis. In the highly ATP demanding, unmyelinated intraocular portions of RGC axons, this OXPHOS reduction may lead to ATP deficiency and cell death. A direct OXPHOS interference would be assumed to be fast and, thus, to be measurable in LHON cyrid cultures. COX activities or respiration rates of EMB treated cells could not be measured directly in the present study with reliable biochemical methods. This approach requires relatively high cell numbers, which were not available, especially not following RA treatment. In contrast, a resulting ATP decline can easily be monitored using minimal amounts of cells, but it must be kept in mind that cybrid cells may rely mainly on glycolytic ATP generation, unless they are forced to switch to the oxidative pathway. Although a short-term RA differentiation of the teratoma based cybrids was applied in the present study, to enhance their OXPHOS dependency, even these cultures may still rely mainly upon glycolytic ATP generation. This may hamper the detection of possible OXPHOS effects of EMB. Moreover, the limited morphological effect of EMB suggests a low efficacy in the model. The problem may be solved by applying the drug to cybrids after terminal neuronal differen-
entiation. Differentiation protocols for the parental cell line have been published [Pleasure et al. 1992], but the cybrids may require astrocytic feeder layers and are expected to offer only low cell yield. Moreover, the required long-term RA treatment seemed to be rather toxic by itself in our hands. The mtDNA copy numbers, determined by Real-time PCR in the present study, were comparable to those found in the same two cell lines earlier, using other mitochondrial and nuclear amplicons [Wong et al. 2002], but did not react to EMB in both cell lines.

If OXPHOS plays no role, other mechanisms may be involved, which could also bring mitochondria onto the scene, since these organelles control the intrinsic apoptotic pathway. One of these mechanisms is glutamate excitotoxicity. EMB seems to require glutamate to reach its maximal toxicity in RGC cultures, which could be diminished by glutamate antagonists [Heng et al. 1999]. Furthermore, the antibiotic enhances the cytosolic and the mitochondrial calcium load [Heng et al. 1999], which is in concordance with the suggested mechanism of glutamate excitotoxicity, including mitochondrial calcium overload, permeability transition and apoptotic neuronal death. On the other hand, LHON mutations can be suggested to facilitate glutamate excitotoxicity. The proposed energy crisis [Carelli et al. 2004a, Zanna et al. 2003] would diminish the available cytoplasmic ATP, which is used by the Na/K-ATPase to generate the plasma membranes resting potential. This would facilitate excitation of NMDA receptors and cytoplasmic calcium increase, which has to be buffered partially by the mitochondrial calcium store. The mutant mitochondria also does not buffer calcium as well as compared to the wild type [Haroon et al. 2007]. In addition, LHON mutations may hamper retinal glutamate clearance by Muller cells, since the mutations diminish glutamate transport activity in cell cultures [Beretta et al. 2004]. It should, thus, be interesting to analyse a possible synergism between EMB, calcium and glutamate in a cybrid model.

**Conclusion**

No synergism between primary LHON mutations and EMB with respect to toxicity, morphological alterations, cellular ATP content and mtDNA copy number could be detected in a teratoma-based LHON cybrid model. Although EMB-induced accumulation of cytoplasmic vacuoles was comparable to earlier observations in RGC cultures, it cannot be ruled out, that the NT2/D1 model may not be ideal to measure EMB induced mitochondrial dysfunction, due to a mainly glycolytic energy metabolism of cybrids. At least it can be stated that EMB is not likely to induce mitochondrial dysfunction in a way which is independent of ETC usage. Moreover, it is unlikely to disturb mitochondrial function and maintenance by inhibiting mtDNA replication.

**Acknowledgment**

We thank Prof. Gino Cortopassi and Prof. Alice Wong for kindly providing the LHON cybrid clone and gratefully acknowledge the excellent technical assistance of Mrs Ines Schellhase. We acknowledge financial support by the NBL grant of the German Ministry for Education and Science (grant number: 01ZZ0407).

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