The Influence of Human N-Acetyltransferase Genotype on the Early Bactericidal Activity of Isoniazid

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(See the editorial commentary by Gillespie on pages 1431–2)

The elimination of isoniazid is subject to the influence of the N-acetyltransferase 2 (NAT2) genotype, and individuals may be homozygotic slow, heterozygotic fast, or homozygotic fast acetylators of isoniazid. The early bactericidal activity (EBA) of an antituberculosis agent can be determined by quantitative culture of Mycobacterium tuberculosis in sputum samples obtained from patients with pulmonary tuberculosis during the first days of treatment. In these studies, the EBA of isoniazid during the first 2 days of treatment was determined for 97 patients with sputum smear–positive pulmonary tuberculosis following isoniazid doses of 37.5 mg, 75 mg, 150 mg, 300 mg, and 600 mg. The NAT2 genotype was determined in 70 patients, and the association between EBA and genotype was examined in this subgroup. Similarly, the relationship between EBA and isoniazid serum concentration was evaluated in 87 patients. The mean EBA of isoniazid increased with dose, but it levelled off between doses of 150 mg (mean EBA, 0.572) and 300 mg (mean EBA, 0.553). Significant differences were found in the mean EBA of isoniazid between the homozygous slow acetylator group and the heterozygous fast acetylator group and between the homozygous slow acetylator group and the homozygous fast acetylator group, but not between the heterozygous fast acetylator group and the homozygous fast acetylator group. The EBA appeared to reach a maximum at a 2-h isoniazid concentration of 2–3 μg/mL.

These data confirm a significant effect of NAT2 genotype on the EBA of isoniazid over a range of doses.

Isoniazid, 50 years after its introduction as an antituberculosis agent, is still a cornerstone of all first-line antituberculosis regimens. It is valued for its wide therapeutic margin, exceptional bactericidal activity, and relatively low toxicity, and it is a key agent in preventing drug resistance [1, 2]. Isoniazid is eliminated via acetylation by the polymorphic N-acetyltransferase 2 (NAT2) enzyme system [3]. Individuals are homozygotic slow (SS), heterozygotic fast (FS), or homozygotic fast (FF) acetylators of isoniazid [4]. Although exposure of SS acetylators to isoniazid is considerably greater than that of faster acetylators, this is not considered of clinical importance, except in individuals receiving once-weekly intermittent treatment [5].

The early bactericidal activity (EBA) of an antituberculosis agent is arbitrarily defined as the decrease during the first 2 days of treatment in log10 colony-forming units (cfu) of Mycobacterium tuberculosis in the sputum of patients with pulmonary tuberculosis confirmed by microscopic examination of smear-positive sputum samples [6]. It reflects an agent’s ability to kill metabolically active organisms present in the walls of tuberculosis cavities [7]. Isoniazid has consistently had the highest EBA of any current antituberculosis agent, and any combination of agents that in-
cludes isoniazid has a similarly high EBA [2, 8]. In an earlier dose-ranging study of isoniazid [9], the EBA was related to the NAT2 genotype in a subset of patients, and FF and FS acetylators had a lower EBA than did SS acetylators who received an identical isoniazid dose. Our report expands these findings and describes the relationship between EBA, isoniazid dose, and the plasma concentrations of isoniazid determined 2, 3, 4, and 5 h after administration of a range of isoniazid doses.

METHODS

Patients. Patients were aged from 18 through 60 years, had never previously received treatment for tuberculosis, and had uncomplicated smear-positive tuberculosis confirmed by microscopic examination of a sputum sample. Patients were recruited from tuberculosis clinics in the Western Cape province of South Africa and were admitted to either the Tygerberg Academic Hospital (Tygerberg, South Africa) or to the Brooklyn Hospital for Chest Diseases (Cape Town, South Africa). Urine samples obtained from the patients were tested for the presence of isoniazid before enrollment in the studies [10]. After discharge from the hospital, the patients immediately commenced therapy with a regimen of isoniazid, rifampin, pyrazinamide, and ethambutol.

Isoniazid treatment regimens. The data presented in our report are derived from 3 studies. In the first study [9] (study 1), published elsewhere, patients admitted to Tygerberg Academic Hospital were randomized to receive isoniazid doses that ranged from 9 mg to 600 mg, and the NAT2 genotype was determined in 37 patients. In 3 patients who received a 9-mg dose of isoniazid and in 7 patients who received a 600-mg dose of isoniazid, serum concentrations of isoniazid were determined at 2, 3, 4, and 5 h after dosing.

In a second study (study 2), 62 patients were admitted to Tygerberg Academic Hospital and randomized to receive isoniazid at a dose of either 6 mg/kg (13 patients), 3 mg/kg (18 patients), or 1.5 mg/kg (18 patients). The results obtained for 13 patients (21%) were excluded from analysis: in 5 of these patients, isoniazid was present in a urine specimen before the first isoniazid dose was administered; the cultures of samples from 4 patients showed no growth, and those from 2 patients showed very poor growth; organisms in a sample obtained from 1 patient were isoniazid resistant; and the initial sputum specimen obtained for 1 patient was destroyed in a laboratory accident. Thirteen of the remaining 49 patients received a 6-mg/kg dose of isoniazid, 18 received a 3-mg/kg dose, and 18 received a 1.5-mg/kg dose. In all 49 patients, isoniazid serum concentrations were determined, and in 41 patients, the NAT2 genotype was determined.

In a third, recently published randomized study of the EBA of streptomycin [11] (study 3), a positive control group of 12 patients each received a 6-mg/kg dose of isoniazid. These patients were admitted to the Brooklyn Hospital for Chest Diseases, located ~20 km from Tygerberg Academic Hospital, but were drawn from the same clinics as those patients enrolled in studies 1 and 2. One patient did not produce a sufficient volume of sputum and was excluded from analysis. Isoniazid serum concentrations were determined in all 11 remaining patients, and the NAT2 genotype was determined in 7 patients.

Sputum collection. Patients were encouraged to cough, and all sputum expectorated between 4 p.m. and 8 a.m. the next morning was collected in wide-mouthed sputum jars. After the first sputum sample (S1) was obtained, the first dose of isoniazid was given. After the second dose of isoniazid was administered, another overnight sputum specimen (S3) was collected. Sputum samples that were <10 mL, heavily blood stained, or diluted by saliva were not analyzed. For each of the 3 studies, all of the microbiological investigations and the determination of isoniazid serum concentrations were performed in the same microbiology and pharmacology laboratories, situated within the Tygerberg Academic Hospital complex.

Microbiological methods. Sputum specimens that could not be delivered immediately were placed on ice, and all samples were delivered to the laboratory within 48 h after they were obtained. Specimens were examined by means of conventional smear and culture for species identification and for determination of resistance to isoniazid. The results for patients with isoniazid-resistant organisms were excluded from final analysis. The sputum was homogenized by stirring with a magnetic follower rod coated with Teflon (DuPont) for 30 min. Ten mL of homogenate was added to an equal volume of dithiothreitol (Sputolysin; Hoechst) in a 50-mL screwtop tube containing 3–6 glass beads and was vortex-mixed for 20 s. The specimen was mixed for an additional 30 min by mechanical shaking, and 2 series of 10-fold dilutions were prepared in distilled water. From each dilution, aliquots of 100 μL were spread onto half plates of 7h11 oleic acid albumin agar medium made selective by the addition of Selectatabs (Mast), to give final concentrations as follows: polymyxin B sulphate, 200,000 U/L; carbenicillin, 100 μg/mL; trimethoprim, 10 μg/mL; and amphotericin B, 100 μg/mL. These plates were placed in a polythene bag together with a plate inoculated with Mycobacterium phlei to provide CO2, and were incubated for 3 weeks. CFUs were counted at that dilution, permitting the counting of 20–200 colonies.

Isoniazid serum concentrations and pharmacokinetic parameters. Isoniazid was administered as crystalline powder (Fluka) with a glass of water after an overnight fast; a light breakfast was allowed after the first blood specimen was drawn 2 h after dosing. Blood samples for determination of isoniazid concentrations were collected in EDTA-coated tubes at 2, 3, 4, and 5 h after the second dose of isoniazid was administered, were immediately placed on ice, and were delivered to the...
laboratory within 5 h after dosing. All samples were analyzed on the same day.

Isoniazid concentrations were determined using the high-performance liquid chromatography (HPLC) methodology of Seifart et al. [12]. Analysis was performed with use of a Hewlett Packard 1090 L HPLC with a UV light detector, set at 340 nm, and an HP 3392 reporting integrator. Separation was on a Whatman Partisol 5 C8 250 mm column (internal diameter, 4.6 mm) maintained at 50°C with a flow rate of 1 mL/min. The mobile phase was composed of a mixture of 50 mmol/L of KH2PO4 (solvent A) in water and 1 volume part of isopropanol in 4 volume parts of acetonitrile (solvent B). The ratio of solvent A to solvent B was altered linearly over the 16-min time course of a run, from 60:40 at the initiation of the run to 30:70 at the end. Analysis of isoniazid over the linear range of 0.5 μg/mL to 25 μg/mL (r = 0.99; n = 10 per data point) showed a coefficient of variation of 1.98% and recovery from biological fluid of 100.01% (SD, 1.13%).

For analysis of each batch of samples, a fresh calibration curve was set up with 5 freshly prepared standards within the range of 0.5–25 μg/mL. In no instance did standards deviate from the existing curve by >5%. The area under the curve (AUC) over the interval of 2–5 h after the dose was calculated by the log linear trapezoidal rule.

**NAT2 genotyping of patients.** A 5-mL blood sample was collected in EDTA-vacutainer tubes from each patient for determination of the NAT2 genotype profile [4]. Genomic DNA (gDNA) was extracted from blood samples using a salting-out procedure [13] and a 1000–base pair (bp) sequence, coding for the NAT2 enzyme, was amplified by PCR. Each 100-μL PCR reaction volume contained 250 ng of gDNA, 0.2 mmol/L of dNTP mixture, 2.5 mmol/L of MgCl2, 0.3 mmol/L primers, 1 X PCR buffer, and 1.25 U of Taq Polymerase I (Invitrogen). The 1000-bp segment was then analyzed by restriction fragment–length polymorphism using 6 enzymes for appropriate cleavage (BamHI, KpnI, MpiI, TaqI, DdeI, and FokI). The NAT2 DNA cleavage profiles yielded by each of the restriction enzymes were analyzed by gel electrophoresis using MetaPhor agarose (BioWhittaker) and were visualized using GelStar nucleic acid stain (BioWhittaker).

**Statistical evaluation.** EBA is here defined as EBA = \((Z_1 - Z_3)/2\), where \(Z_1\) and \(Z_3\) are logarithms of the cfu count per mL of sputum obtained during the S1 and S3 collections, respectively. For the purpose of analysis, the patients who received a 6-mg/kg, 3-mg/kg, or 1.5-mg/kg dose of isoniazid were grouped with those who received a 300-mg, 150-mg, or 75-mg dose of isoniazid, respectively. Those patients who received a dose of ≤37.5 mg were analyzed as 1 group. The mean values, median values, and SDs of \(Z_1\), \(Z_3\), \((Z_1 - Z_3)\) and the EBA of isoniazid for the dosage groups and subgroups formed within the dosage groups by NAT2 genotyping were calculated.

For investigation of the relation between EBA and the 2 factors of dose level and genotype, the results of the studies were pooled. The statistical justification for pooling was by formal comparisons of mean EBA values using analysis of variance techniques, as detailed in Results.

To examine the association of EBA with the 2 factors of dose level and genotype and allow for the possible influence of the log10 cfu count for the S1 sample, a linear model of the form

\[ Y_{ik} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma x_k + \text{random error} \]

was fitted by the method of least squares. In the model, the response \(Y_{ik}\) is EBA, \(\mu\) is an overall mean value, \(\alpha_i\) is the dose level effect, \(\beta_j\) is the genotype effect, \((\alpha\beta)_{ij}\) is the dose level/genotype interaction, and \(\gamma\) is the regression coefficient of the log10 cfu count for the S1 sample value \(x_k\). The significances of the various effects were tested by analysis of variance on the basis of this model. The interaction effect and the influence of the log10 cfu count for the S1 sample were not significant, so the final model used was

\[ Y_{ik} = \mu + \alpha_i + \beta_j + \text{random error} \]

and the least squares means are \(\mu + \alpha_i\) and \(\mu + \beta_j\). The SEs of the least squares means and of contrasts between them are derived from the covariance matrix of estimates of parameters in the model. The results of least squares analysis were confirmed by analysis on the basis of the ranks described in Hettmansperger [14] and implemented in the program Minitab 10Xtra [15].

Each study was approved by the Institutional Review Board of the Faculty of Health Sciences of Stellenbosch University. Informed written consent for participation in the studies was obtained from each patient before enrollment.

**RESULTS**

Results are presented for 97 patients (mean age, 31.1 years; mean weight, 51 kg). Ninety-four patients (97%) had multicavitary disease involving both lungs that was visible on chest radiograph. Table 1 summarizes data on the age, sex, and weight of the patients; the radiographic extent of disease; the degree of sputum smear positivity; and the mean log10 cfu counts per mL of sputum for each of the 3 studies. There were no significant differences between patients enrolled in different studies, with exception differences in the degree of sputum smear positivity and in the cfu counts for the S1 sputum sample. With regard to cfu counts for the S1 sample, a 1-way analysis of variance test of study effect gave the result F\(_{2,94} = 2.81\) (\(P = .065\)). Testing the mean log10 cfu count for study 1 against the mean values for studies 2 and 3 gave F\(_{2,95} = 5.29\) (\(P = .024\)). To allow for the influence of S1 cfu counts on EBA, log10S1
Table 1. Demographic and clinical characteristics of patients in 3 different studies of isoniazid treatment.

| Characteristic                                      | Study 1 [9] (n = 37) | Study 2 [10] (n = 49) | Study 3 [11] (n = 11) |
|----------------------------------------------------|----------------------|-----------------------|-----------------------|
| Age, mean years                                    | 29.5                 | 31.1                  | 36.1                  |
| Weight, mean kg                                    | 50.3                 | 51.3                  | 52.1                  |
| Male sex, % of patients                            | 65                   | 69                    | 73                    |
| Disease in both lungs, % of patients                | 85                   | 94                    | 91                    |
| Multicavitary disease, % of patients                | 94                   | 98                    | 100                   |
| Sputum samples strongly positive (3+) on microscopy for AFB, % of patients<sup>a</sup> | 28                   | 73                    | 73                    |
| Initial sputum sample, log<sub>10</sub> cfu/mL<sup>b</sup> | 6.789                | 7.058                 | 7.181                 |

NOTE. Study 1 [9] was an isoniazid dose-ranging study. Study 2 was a randomized study of 3 different doses of isoniazid. Study 3 [11] was a study of the early bactericidal activity of streptomycin. AFB, acid-fast bacilli.

was introduced as covariate in the analysis of association between EBA and the factors dose level and genotype. The effect of S1 log<sub>10</sub> cfu count was not significant.

For detailed analysis of the effects of the factors dose level and genotype on EBA, the results of the 3 studies were pooled. The justification for pooling these data was that no significant differences between mean EBA levels were found at dose levels for which direct comparisons were possible. The mean EBA values derived from studies 1 and 2 were compared at the 75-mg dose level and the 150-mg dose level. At the 75-mg dose level, the mean EBA value (±SD) for study 1, based on data obtained from 3 patients, was 0.227 ± 0.231; the mean EBA value (±SD) for study 2, based on data obtained from 18 patients, was 0.302 ± 0.272. At the 150-mg dose level, the mean EBA value (±SD) for study 1, based on data obtained from 3 patients, was 0.360 ± 0.110; the mean EBA value (±SD) for study 2, based on data obtained from 18 patients, was 0.552 ± 0.300. A 2-way analysis of variance showed that the interaction effect was not significant (F<sub>1,80</sub> = 0.23; P = .63). The effect of study was not significant (F<sub>3,90</sub> = 1.22; P = .28). The mean EBA values derived from studies 2 and 3 were compared at the 300-mg dose level; the mean EBA value (±SD) for study 2, based on data obtained from 13 patients, was 0.617 ± 0.243, and the mean EBA value (±SD) for study 3, based on data obtained from 11 patients, was 0.515 ± 0.200. The mean EBA values were not significantly different (F<sub>2,22</sub> = 1.22; P = .28).

Table 2 summarizes the mean EBA values in relation to genotype and dose level. Analysis of variance resulting from fitting the linear model revealed that the effect of covariate S1 log<sub>10</sub> cfu count was not significant (F<sub>1,71</sub> = 1.72; P = .194). The dose level/

Table 2. Influence of dose and N-acetyltransferase genotype on early bactericidal activity (EBA) of isoniazid.

| Isoniazid dose, mg | Homozygous slow acetylator (SS) group | Heterozygous fast acetylator (FS) group | Homozygous fast acetylator (FF) group | Least squares mean EBA | Least squares mean SE |
|-------------------|---------------------------------------|----------------------------------------|---------------------------------------|------------------------|-----------------------|
|                   | No. of patients | Mean EBA | No. of patients | Mean EBA | No. of patients | Mean EBA | No. of patients | Mean EBA |
| ≤37.5             | 8            | 0.111   | 7            | 0.009   | 5            | −0.093  | 0.054         | 0.007   |
| 75                | 9            | 0.379   | 9            | 0.290   | 1            | 0.298   | 0.058         | 0.255   |
| 150               | 4            | 0.824   | 9            | 0.479   | 3            | 0.532   | 0.061         | 0.572   |
| 300               | 6            | 0.606   | 5            | 0.467   | 10           | 0.523   | 0.053         | 0.553   |
| 600               | 1            | 0.686   | 5            | 0.695   | 5            | 0.352   | 0.074         | 0.582   |
| All               | 28           | 0.514<sup>a</sup> | 36          | 0.383<sup>b</sup> | 23          | 0.284<sup>c</sup> | ... | ... |

NOTE. Pairwise comparisons of least squares mean EBA in the different genotype groups are as follows: for SS group versus FS group, the difference (±SE) is 0.131 ± 0.062 and the t<sub>9</sub> is 2.11; for SS group versus FF group, the difference (±SE) is 0.230 ± 0.071 and the t<sub>10</sub> is 3.23; for FS group versus FF group, the difference (±SE) is 0.099 ± 0.067 and the t<sub>22</sub> is 1.47.

<sup>a</sup> Mean SE, 0.048.
<sup>b</sup> Mean SE, 0.041.
<sup>c</sup> Mean SE, 0.052.
genotype interaction effect was also not significant (F_{4,71} = 1.47; \( P = .185 \)). Consequently, the reduced model with only the main effects of the factors dose level and genotype was fitted. The test for dose level effect gave \( F_{2,71} = 19.58 \) (\( P < .0005 \)). The test for genotype effect gave \( F_{3,71} = 5.40 \) (\( P = .006 \)).

The least squares mean EBA (±SE) of isoniazid was 0.514 ± 0.048 for the SS genotype, 0.383 ± 0.04122 for the FS genotype, and 0.284 ± 0.052 for the FF genotype. Although the results of the F-test given above show clear lack of homogeneity of these means, the pairwise comparisons given in the note to Table 2 are used to indicate more precisely where the differences occur. The mean EBA of isoniazid for those with the SS genotype is clearly separated from that of those with the FF genotype and, to a lesser extent, that of those with the FS genotype, but the mean EBAs of isoniazid for the FS and FF genotype groups cannot be separated.

The dose-level least squares mean EBAs are given in Table 2 together with SEs. According to the analysis of variance F-test, there were significant differences between the means. To identify where the differences occur, some pairwise comparisons are useful. The difference between the mean EBA values (±SE) at the 75-mg and 150-mg dose levels is 0.248 ± 0.078, giving a \( t_m \) of 3.18 and a \( P \) value of .002, which is clearly significant. The difference between the mean EBA values (±SE) at the 150-mg and 75-mg dose levels is 0.317 ± 0.083, giving a \( t_m \) value of 3.82 and a \( P \) value of <.0005, also clearly significant. The 95% CIs can easily be calculated for the least squares means by adding and subtracting 1.99 multiplied by the SE. Such calculations provide an approximate check of the pairwise significance tests, and they also show clearly that there are no significant differences between the mean EBA values at the 150-mg, 300-mg, and 600-mg dose levels.

Figure 1 illustrates a significant relationship between exposure to isoniazid, as reflected in log AUC for the period 2–5 h after isoniazid administration, and EBA. The fitted line is EBA = 0.284 + 0.140 log AUC, and the ratio of the slope coefficient to its standard error (\( t \)) was 5.43 (\( P < .001 \)). Fitting a straight-line EBA on 2-h, 3-h, 4-h, or 5-h isoniazid concentrations gives very similar results. Table 3 gives the mean isoniazid concentrations achieved 2 h after isoniazid dosing and the resulting mean EBA values in the same patients. Above a 2-h mean isoniazid concentration of ∼2.5 µg/mL, no further increase in EBA occurs. It is noteworthy, in this respect, that 50% of patients who were FF acetylators of isoniazid and who received a conventional 300-mg (6-mg/kg) dose of isoniazid had a 2-h serum concentration of isoniazid of <3 µg/mL, and 22% of such patients had a concentration of <2 µg/mL.

**DISCUSSION**

Within the window provided by the first 2 days of treatment, our results show that the EBA of isoniazid is influenced by the NAT2 genotype and by the serum concentrations of isoniazid. Nonetheless, the EBA achieved by both the FS and FF acetylators of isoniazid who received 300-mg doses of isoniazid was substantially higher than that achieved with any other first line agent [6, 8].

EBA was also related to the isoniazid AUC for the period 2–5 h after administration of the dose and was similarly related to the mean isoniazid concentration at 2, 3, 4, and 5 h after dosing. Using the isoniazid concentration at 2 h after dosing as a convenient reference point (frequently quoted in the literature), a maximum isoniazid EBA was reached at a 2-h isoniazid concentration of 2–3 µg/mL; further increases in isoniazid concentration, as with increases in dose, did not increase the EBA. In vitro studies of exposure of proliferating bacilli to isoniazid concentrations of 1–2 µg/mL found a decrease in viable cfu counts to ∼1% of the original population within 3–4 days. This in vitro killing was also dependent on active mycobacterial growth and, above a certain optimum level, was also concentration independent [16]. Our results also lend sup-

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**Table 3. Early bactericidal activity (EBA), by isoniazid serum concentrations 2 h after isoniazid dosing.**

| Isoniazid serum concentration (µg/mL) | No. of patients | Isoniazid concentration (mean µg/mL) | Mean EBA     |
|--------------------------------------|-----------------|--------------------------------------|--------------|
| 0.0–0.5                              | 6               | 0.25                                 | -0.034       |
| 0.5–1.0                              | 12              | 0.77                                 | 0.342        |
| 1.0–1.5                              | 4               | 1.20                                 | 0.455        |
| 1.5–2.0                              | 9               | 1.70                                 | 0.390        |
| 2.0–3.0                              | 10              | 2.54                                 | 0.609        |
| 3.0–6.0                              | 18              | 4.07                                 | 0.633        |
| > 6.0                                | 11              | 9.84                                 | 0.526        |
port to the designation of a 2-h isoniazid concentration of \(~3 \mu g/mL\) as the lower limit of the normal range [17].

With supervised combination therapy, a deficiency in optimal killing of actively metabolizing bacilli—such as was shown to be the case for the patients who were faster acetylators of isoniazid in this study—may well be unimportant for the final outcome of treatment. Our results also refer specifically to the first 2 days of treatment, when large numbers of actively metabolizing organisms are present in lung cavities and the greatest risk exists for the selection of drug-resistant mutants. Actively metabolizing, fully drug-susceptible organisms are easily killed by relatively low concentrations of isoniazid, as is demonstrated by this study, and the isoniazid-resistant mutants should be killed by the companion drugs (rifampin, ethambutol, and, to a lesser extent, pyrazinamide). As these active organisms are killed, the activity of isoniazid also declines [6]. At this point, rifampin and, later, pyrazinamide become the most active agents in the standard antituberculosis regimen, and isoniazid is relegated to a relatively minor supporting role [2]. Later regrowth of bacilli due to “functional monotherapy” or “cryptic” or overt noncompliance with therapy may, however, allow the recreation of the situation that existed at the start of treatment [18]. The deficiencies in bactericidal activity caused by exposure to lower concentrations of isoniazid may then again become relevant. The vulnerability of patients who are FF acetylators during intermittent therapy (which could be considered as a model for noncompliance) was recently demonstrated again [19]. Even a small reduction in isoniazid dosage during daily therapy may be associated with decreased therapeutic efficacy [20].

In conclusion, our data confirm the exceptional bactericidal activity of isoniazid when acting on fully drug-susceptible organisms during the first 2 days of treatment. Although this activity is significantly influenced by the NAT2 genotype, even in individuals who are FF acetylators of isoniazid, a relatively high EBA is still reached at doses that would usually be used in clinical practice.

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