Research Article

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Benzo(a)pyrene degradation pathway in *Bacillus subtilis* BMT4i (MTCC 9447) *Bacillus subtilis* BMT4i'deki (MTCC 9447) Benzo(a)piren Bozunma Yolu

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Abstract

Background: Benzo(a)pyrene (BaP), a high molecular weight pentacyclic aromatic hydrocarbon, is a priority pollutant of extreme concern. *Bacillus subtilis* BMT4i (MTCC 9447) degrades BaP through chromosomally encoded pathway. Nevertheless, inadequate information is available on BaP degradation pathway in genus *Bacillus* despite of its species being shown as potent BaP degrader. The objective of this study was to elucidate BaP degradation pathway in *B. subtilis* strain BMT4i by identifying metabolites through UHPLC-MS.

Materials and methods: Batch experiments were conducted to characterize metabolic pathway of BaP in the bacterium *B. subtilis* BMT4i. The metabolites were separated and characterized by UHPLC-MS.

Results: The major intermediates of BaP metabolism that had accumulated in the culture media after 15 days of incubation were benzo(a)pyrene-11,12-epoxide, 7,8,9,10-tetrahydrobenzo[*pqr*]tetraphene-7,8,9,10-tetraol, benzo(a)pyrene-*cis*-7,8-dihydrodiol, 8-carboxy-7-hydroxy pyrene, chrysene-4 or 5-carboxylic acid, *cis*-4-(8-hydroxypyrene-7yl)-2-oxobut-3-enoic acid, hydroxymethoxybenzo(a)

pyrene and dimethoxybenzo(a)pyrene. Among above, 8-carboxy-7-hydroxy pyrene, chrysene-4 or 5-carboxylic acid, and *cis*-4-(8-hydroxypyrene-7yl)-2-oxobut-3-enoic acid are ring cleavage products of BaP.

Conclusion: The identified metabolites indicated that BMT4i initially oxidized BaP with monooxygenases and dioxygenases at C-11,12 or and C-7,8 and C-9,10 positions, suggesting operation of multiple pathways for BaP degradation in *B. subtilis*. Further studies are essential to find out whether the entire biodegradation process in *B. subtilis* results into metabolic detoxification of BaP or not.

Keywords: Benzo(a)pyrene degradation; *Bacillus subtilis*; Benzo(a)pyrene-*cis*-7,8-dihydrodiol; Dioxygenase; Monooxygenase.

Özet

Amaç: Yüksek moleküler ağırlıklı beş halkalı bir aromatik hidrokarbon olan benzo(a)piren (BaP), son derece ilgi çeken önemli bir kirlenici maddedir. *Bacillus subtilis* BMT4i (MTCC 9447) BaP'ı kromozomal olarak kodlanmış yol ile bozar. Bununla birlikte, türleri güçlü BaP bozuncusu olarak gösterilmesine rağmen, *Bacillus* cinsinin BaP bozunma yolunda yetersiz bilgi mevcuttur. Bu çalışmanın amacı *B. subtilis* suşu BMT4i'de BaP yıkım yolunu, UHPLC-MS ile metabolitleri tanımlayarak, aydınlatmaktır.

Gereç ve yöntem: *Bacillus subtilis* BMT4i bakterisinde BaP'nin metabolik yolunu karakterize etmek için bir grup deney yapıldı. Metabolitler UHPLC-MS ile ayrıldı ve karakterize edildi.

Bulgular: 15 günlük inkübasyondan sonra kültür ortamında biriken BaP metabolizmasının başlıca ara maddeleri, benzo(a)piren-11,12-epoksit, 7,8,9,10-tetrahydrobenzo[*pqr*] tetrafen-7,8,9,10-tetraol, benzo(a)piren-*cis*-7,8-dihidrodiol, 8-karboksi-7-hidroksi piren, chrysene-4 veya 5-karboksilik asit, *cis*-4-(8-hidroksipiren-7-il)-2-oksobut-3-enoik

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asit, hidroksimetoksibenzo(a)piren ve dimetoksibenzo(a)piren. Yukardakiler arasında, 8-karboksi-7-hidroksi piren, chrysene -4 veya 5-karboksilik asit ve cis-4- (8-hidroksi-piren-7-il) -2-oksobut-3-enoik asit BaP'nin halka bölünme ürünleridir.

Sonuç: Tanımlanan metabolitler BMT4i'nin başlangıçta BaP'ı C-11,12'de monoksijenazlar ve dioksijenazlarla veya C-7,8 ve C-9,10 pozisyonlarından okside ettiğini ve *B. subtilis*'de BaP yıkımı için çoklu yolların çalışıyor olabileceğini göstermiştir. *Bacillus subtilis*'deki tüm biyodegradasyon sürecinin BaP'nin metabolik detoksifikasyonuna yol açıp açmadığını anlamak için ileri çalışmalar gereklidir.

Anahtar sözcükler: Benzo(a)piren yıkımı; *Bacillus subtilis*; Benzo(a)piren-cis-7,8-dihidrodiol; Dioksijenaz; Monooksijenaz.

Introduction

Benzo(a)pyrene (BaP), a high molecular weight pentacyclic aromatic hydrocarbon, is a priority pollutant found in air, water, and soil [1–3]. It is highly recalcitrant to microbial degradation due to its low aqueous solubility and thermodynamic stability of its fused ring structure [4–6]. US Environmental Protection Agency (USEPA) has categorized BaP as a priority pollutant of extreme concern in view of its cytotoxicity, carcinogenicity, and genotoxicity [7–9]. Microorganism mediated BaP degradation may play a central role in the decontaminating sediment and surface soils [10, 11]. Few bacterial species namely *Mycobacterium* sp. [12–14], *Sphingomonas paucimobilis* [15], *Stenotrophomonas maltophilia* [16, 17], *Mycobacterium vanbaalenii* Pyr-1 [18], have been reported to degrade BaP cometabolically while *Bacillus subtilis* BMT4i (MTCC 9447) has been reported to be a potent degrader of BaP as sole carbon and energy source [19].

BaP degradation has been extensively studied in bacteria suggesting execution of multiple degradation pathways on the basis of identified metabolites. BaP oxidation to dihydrodiols has been reported in *Sphingomonas yanoikuyae* B8/36 excluding any ring cleavage products [20]. BaP degradation in *Mycobacterium* sp. strain RJGII-135 yielded benzo(a)pyrene-7,8-dihydrodiol and three ring-cleavage products [14]. *Mycobacterium vanbaalenii* PYR-1 degraded BaP to several dihydrodiols and one ring-cleavage product, 10-oxabenzo [def] chrysene-9-one [18]. Growth of *S. yanoikuyae* JAR02 on BaP yielded benzo(a)pyrene-cis 7,8-dihydrodiol and benzo(a)pyrene-cis 9,10-dihydrodiol in addition to pyrene-8-hydroxy-7-carboxylic acid and pyrene-7-hydroxy-8-carboxylic acid as novel ring-cleavage

metabolites [21]. These reports suggested involvement of dioxygenase and monooxygenase which incorporate two or one oxygen atoms into the aromatic nucleus forming *cis*-dihydrodiols and *trans*-dihydrodiols, respectively as initial ring cleavage enzymes in BaP degradation pathway.

However, limited information is available on BaP degradation pathway in genus *Bacillus* despite of its species being shown as potent BaP degraders [19, 22, 23]. A bacterial consortium of *Bacillus cereus* and *Bacillus vireti* isolated from petrochemical soil has been reported to degrade BaP into *cis*-4-(8-hydroxy-pyren-7-yl)-2-oxobut-3-enoic acid, a ring cleavage product of 9,10-dihydrodiol [23]. We have shown previously that *B. subtilis* BMT4i (MTCC 9447) degrades BaP very efficiently up to 84.66% in 28 days via chromosomally encoded pathway [19, 22].

As an extension of previous study, the present work is performed to elucidate BaP degradation pathway in *B. subtilis* strain BMT4i by identifying metabolites through Ultra High Performance Liquid Chromatography-Mass Spectroscopy (UHPLC-MS). The present study reports the presence of eight different metabolites including ring cleavage products suggesting functioning of multiple degradation pathways involving dioxygenases and monooxygenase as the initial attacking enzymes.

Materials and methods

Chemicals and reagents

The BaP (99.9%) was purchased from Sigma-Aldrich Pvt. Ltd. Missouri, MO, USA. Metabolite standards for benzo(a)pyrene-cis-4,5-dihydrodiol, benzo(a)pyrene-cis-7,8-dihydrodiol were acquired from the NCI Chemical Carcinogen Reference Standards Repository (Kansas, Missouri, MO, USA). HPLC-grade methanol, acetonitrile, dimethylformamide, formic acid were purchased from Merck Pvt. Ltd., Maharashtra, India. Nutrient Agar media and broth were purchased from Himedia Mumbai, Maharashtra, India. The general chemicals including constituents of basal salt mineral media (BSM) and solvents of analytical grade were purchased from Glaxo SmithKline Pvt. Ltd., Mumbai, Maharashtra, India and Merck Life Science Pvt. Ltd., Bengaluru, Karnataka, India.

Identification of metabolites of BaP degradation in *Bacillus subtilis* BMT4i

For the identification of BaP degradation metabolites, 100 mL BSM culture of *B. subtilis* BMT4i (10^7 cells/mL)

containing BaP (50 µg/mL) in amber bottle was incubated at 30°C for 40 days in triplicate. At various time intervals (7, 15, and 40 days), 25 mL culture broth was withdrawn and 100 µL of the same was checked for BMT4i growth by CFU method [19]. Remaining withdrawn culture was acidified (pH 2.5) and processed for the recovery of products by ethyl

acetate extraction [21]. The extracts were dried using rotary evaporator and dissolved in 5 mL of methanol. The organic extracts were analyzed using UHPLC-MS analysis on commercial basis from Sophisticated Analytical Instrument Facility (SAIF), India Institute of Technology, Mumbai, Maharashtra, India. Identification of metabolites was

Figure 1: Out of eight peaks, peak I was exclusively found in 15-day sample.

UHPLC chromatograms showing benzo(a)pyrene and its metabolites after (A) 7 day (B) 15 day (C) 40 day.

done by comparing the mass spectral data of metabolites obtained with that of commercially available BaP metabolite standards and those reported in the literature [18, 21].

UHPLC-MS Analysis

Stock solutions of BaP and metabolite standards were prepared at concentration of 5 mg/mL in dimethylformamide since the compounds were readily soluble in

dimethylformamide. In addition, working stock solution of the same was prepared in methanol at concentration of 0.2 mg/mL [19]. Furthermore, the standards and samples filtered through glass syringe using 0.2 μ m filter and then analyzed in UHPLC (1290 Infinity Binary pump, Agilent Technologies, Santa Clara, CA, USA) coupled with 6550 i-Funnel Quadrupole time-of-flight (QTOF) mass spectrometer. The mobile phase was a gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile at a flow rate of 0.3 mL/min for 30 min; the injection

Figure 2: Three metabolites namely 8-carboxy-7-hydroxy pyrene, chrysene-4 or 5-carboxylic acid and *cis*-4-(8-hydroxypyrene-7yl)-2-oxobut-3-enoic acid are the ring cleavage metabolites.

Full scan mass spectra of BaP metabolites showing the abundance of (A) BaP standard as mass peak, (B) peak I (benzo(a)pyrene-11, 12 epoxide as $M + Na$ adduct), (C) peak II (7,8,9,10-tetrahydrobenzo[ghi]perylene-7,8,9,10-tetraol as $M + H$), (D) peak III (benzo(a)pyrene-*cis*-7,8-dihydrodiol as dimer $M + H$), (E) peak IV (8-carboxy-7-hydroxy pyrene as $M + CH_3CN + H$), (F) peak V (chrysene-4 or 5-carboxylic acid as mass peak), (G) peak VI (*cis*-4-(8-hydroxypyrene-7yl)-2-oxobut-3-enoic acid as mass peak), (H) peak VII (hydroxymethoxybenzo(a)pyrene as $M + H$) and (I) peak VIII (dimethoxy-benzo(a)pyrene as $M + H$ and benzo(a)pyrene as mass peak).

Figure 2 (continued)

volume was 3 μ L. The column used was Zorbax SB C18 Rapid Resolution HD, 2.1×100 mm, 1.8 μ m, (Agilent Technologies, Santa Clara, CA, USA). The nitrogen gas flow was 13 mL/min at 25°C, and the sheath gas flow was 11 mL/min at 30°C with a nebulizer pressure at 35 psi. The capillary voltage was 3500 V with a nozzle voltage of 1000 V. Fragmentation energy was kept at 175 V. The analysis was done in both positive and negative ESI mode, the data was acquired in Agilent Masshunter Data Acquisition software (Version B.05.00) (Agilent Technologies, Santa Clara, CA, USA) and the data were analyzed in Agilent Masshunter Qualitative Software (Version B.06.00) (Agilent Technologies, Santa Clara, CA, USA).

Results and discussion

Identification of metabolites

Metabolic intermediates formed as a result of BaP degradation were recovered and subjected to UHPLC-MS analysis for identification. Firstly, the standards of commercially available metabolite were analyzed to identify the corresponding fragments. Each standard was injected separately, and its mass spectra and m/z values were obtained. The fragmentation pattern of individual

metabolite standards and of a mixture of metabolite standards was obtained. Comparison of fragmentation pattern led to the confirmation of the presence of a particular metabolite. MS data (mass of recovered metabolites and their fragmentation pattern) of individual peaks were also compared with the MS data of metabolites of BaP biodegradation reported in the literature [18, 21].

UHPLC-MS analysis of 15-day sample revealed the presence of several peaks out of which eight peaks were identified (Figures 1A–C and 2; Table 1). Peaks II–VIII were observed in 7-day sample in addition to 15-day. However, peak I was exclusively found in 15-day sample. Peaks III–VI were also observed in 7 and 40 day samples in addition to 15 day samples, for peak I, (retention time 3.982 min) the characteristic mass fragment was m/z of 289.124 $[M+Na]$. The m/z of 289.124 $[M+Na]$ of peak I compound was similar to the benzo(a)pyrene-11,12-epoxide (mol. wt. 266.29), an unstable intermediate formed due to action of cytochrome P450 on BaP which after subsequent hydrolysis converted into benzo(a)pyrene-*trans*-11,12-dihydrodiol in *M. vanbaalenii* PYR-1 [18]. Peak II eluted at 6.41 min. Mass fragment of peak II was m/z of 321.129 $[M+H]$ which corresponded to compound 7,8,9,10-tetrahydrobenzo[*pqr*]-tetraphene-7,8,9,10-tetraol (mol. wt. 320.33) shown to be generated in filamentous fungi *Cunninghamella elegans* by cytochrome-P450 monooxygenase during BaP degradation [24]. Although this kind of compound has not yet

Table 1: BaP metabolites' structure, molecular formula, m/z peaks, sample and elution time.

Identified compound name	Mol. mass	m/z value	Peak	Sample time (Day)/peak	Elution time (min)
Benzo(a)pyrene-11,12-epoxide (C ₂₀ H ₁₀ O)	266.29	289.124	M + Na	15 (Peak I)	3.982
7,8,9,10-tetrahydrobenzo[<i>pqr</i>]tetraphene-7,8,9,10-tetraol (C ₂₀ H ₁₆ O ₄)	320.33	321.129	M + H	7 (Peak II)	6.439
				15 (Peak II)	6.417
Benzo(a)pyrene- <i>cis</i> -7,8-dihydrodiol (C ₂₀ H ₁₄ O ₂)	286.32	573.249	Dimer + H	7 (Peak III)	9.381
				15 (Peak III)	9.396
				40 (Peak III)	9.421
8-carboxy-7-hydroxy pyrene (C ₁₇ H ₁₀ O ₂)	246.26	288.288	M + H + CH ₃ CN	7 (Peak IV)	10.765
				15 (Peak IV)	10.768
				40 (Peak IV)	10.768
Chrysene-4 or 5-carboxylic acid (C ₂₀ H ₁₆ O ₂)	288.11	288.28	Mass peak	7 (Peak V)	11.025
				15 (Peak V)	10.990
				40 (Peak V)	11.015
<i>cis</i> -4-(8-hydroxypyrene-7yl)-2-oxobut-3-enoic acid (C ₂₀ H ₁₂ O ₄)	316.31	316.319	Mass peak	7 (Peak VI)	12.236
				15 (Peak VI)	12.239
				40 (Peak VI)	12.239
Hydroxymethoxybenzo(a)pyrene (C ₂₁ H ₁₄ O ₂)	298.33	299.105	M + 1	7 (Peak VII)	12.755
				15 (Peak VII)	12.733
Dimethoxybenzo(a)pyrene (C ₂₂ H ₁₆ O ₂)	312.36	313.271	M + 1	7 (Peak VIII)	17.736
				15 (Peak VIII)	17.615
Benzo(a)pyrene (C ₂₀ H ₁₂)	252.32	252.099	Mass peak	Standard	17.704

been reported in any bacterial species, however, *Bacillus megaterium* cytochrome-P450 BM3 monooxygenase has been reported to be a unique enzyme which can catalyze a wide range of similar reactions [25]. Peak III eluted at 9.396 min demonstrated mass fragment of m/z of 573.249 (dimer + H). The elution time and mass fragment of peak III was found to be identical with the elution time and mass fragment of the standard metabolite benzo(a)pyrene-*cis*-7,8-dihydrodiol (mol. wt. 286.32) dimer. Therefore, peak III was identified as benzo(a)pyrene-*cis*-7,8-dihydrodiol. Peak IV eluted at 10.768 showed mass fragment of m/z of 288.288 [M + CH₃CN + H]. Its base peak mass (mol. wt. 246.26) was identical to the mass of 8-carboxy-7-hydroxy pyrene (mol. wt. 246.26) which was shown to be a ring cleavage intermediate in BaP degradation pathway in *S. yanoikuyae* JAR02 [21]. Peak V eluted at 10.990 min showed mass peak fragment of m/z of 288.28 [M] which corresponded to compound chrysene-4 or 5-carboxylic acid (mol. wt. 288.11) as reported in BaP degradation in *M. vanbaalenii* PYR-1 [18]. Peak VI was eluted at 12.239 min and its m/z ratio was found to be 316.319 (M). On the basis of its m/z value, it was identified as *cis*-4-(8-hydroxypyrene-7yl)-2-oxobut-3-enoic acid (mol. wt. 316.31) which was earlier found to be a ring cleavage product of benzo(a)pyrene-*cis*-9,10-dihydrodiol in *M. vanbaalenii* PYR-1 [18]. Peak VII with elution time 12.733 showed mass fragment of m/z of 299.105 [M + H] corresponding to the hydroxymethoxybenzo(a)pyrene (mol. wt. 298.33) which was previously reported in *B. cereus*, *B. vireti*

and *M. vanbaalenii* PYR-1 [18, 23]. Peak VIII illustrated presence of two compounds on mass fragmentation. Mass fragment of m/z of 313.271 [M + H] with elution time of 17.615 min was found to be parallel to mass of the dimethoxy-benzo(a)-pyrene (mol. wt. 312.36), and mass fragment having m/z of 252.099 [M] with elution time of 17.704 min corresponded to the mass peak of benzo(a)pyrene standard itself (mol. wt. 252.32). The finding was in accordance with earlier reports on *M. vanbaalenii* PYR-1 mediated BaP degradation [18].

Elucidation of BaP degradation pathway in *Bacillus subtilis* BMT4i

The identification of total eight metabolites produced during BaP degradation led to elucidation of BaP degradation pathway for the first time in *B. subtilis* BMT4i (Figure 3). Out of eight, three metabolites namely 8-carboxy-7-hydroxy pyrene, chrysene-4 or 5-carboxylic acid and *cis*-4-(8-hydroxypyrene-7yl)-2-oxobut-3-enoic acid are the ring cleavage metabolites while others are the intermediates of the degradation pathway prior to ring cleavage. On the basis of identified metabolites, we have proposed BaP biodegradation pathway in *B. subtilis* BMT4i, which is in agreement with the pathways documented in the previous studies [14, 18, 21, 23]. The listed metabolites are known to be generated by various bacterial species such as *Mycobacterium* sp. Strain RJGII-135

Figure 3: Proposed pathways for degradation of BaP by *B. subtilis* BMT4i (MTCC 9447) which is in consonance to BaP degradation pathways proposed by Moody et al. [18]; Rentz et al. [21].

Peak I (benzo(a)pyrene-11, 12 epoxide), peak II (7,8,9,10-tetrahydrobenzo[pqr]tetraphene-7,8,9,10-tetraol), peak III (benzo(a)pyrene-*cis*-7,8-dihydrodiol), peak IV (8-carboxy-7-hydroxy pyrene), peak V (chrysene-4 or 5-carboxylic acid), peak VI (*cis*-4-(8-hydroxypyrene-7yl)-2-oxobut-3-enoic acid), peak VII (hydroxymethoxybenzo(a)pyrene), peak VIII (dimethoxy-benzo(a)pyrene). Metabolites within the box are hypothetical intermediates and have not been measured in this study.

[14], *M. vanbaalenii* PYR-1 [18], and *S. yanoikuyae* JAR02 [21], which suggested functioning of multiple pathways involving dioxygenases and monooxygenase as the initial attacking enzymes in *B. subtilis* BMT4i. Bacteria initially oxidize aromatic hydrocarbons to *cis*-dihydrodiols [16]. The oxidation of these compounds involves the enzymatic incorporation of atmospheric oxygen into the substrate. Characteristically, bacteria produce dioxygenases and mono-oxygenases, which incorporate two or one oxygen atoms into the aromatic nucleus forming *cis*-dihydrodiols and *trans*-dihydrodiols, respectively [16, 26]. The initial ring oxidation is usually the rate-limiting step in the biodegradation reaction of PAHs. Further oxidation of the *cis*-dihydrodiols leads to the formation of catechols which are substrates for other dioxygenases that bring about enzymatic cleavage of the aromatic ring.

As shown in proposed pathway of BaP degradation in Figure 3, benzo(a)pyrene-11, 12-epoxide is the precursor of benzo(a)pyrene-*trans*-11,12-dihydrodiol which is further converted to hydroxymethoxybenzo(a)pyrene subsequently leading to formation of dimethoxybenzo(a)pyrene. The dioxygenase mediated

hydroxylation at 4, 5 or 11, 12 positions in BaP alternatively may generate benzo(a)pyrene-*cis*-4,5-dihydrodiol or benzo(a)pyrene-*cis*-11,12-dihydrodiol, respectively leading to hydroxymethoxybenzo(a)pyrene which is further metabolized to corresponding dimethoxy benzo(a)pyrene [18]. However, at this moment we cannot ascertain the positions of substitution due to lack of substantial data. Formation of tetrahydrobenzo[pqr]tetraphene-7,8,9,10-tetraol might be result of epoxidation of BaP by cytochrome-P450 monooxygenase [24].

Two other accumulated intermediates namely *cis*-4-(8-hydroxypyrene-7yl)-2-oxobut-3-enoic acid and 8-carboxy-7-hydroxypyrene were considered as products of benzo(a)pyrene-*cis*-9,10-dihydrodiol and benzo(a)pyrene-*cis*-7,8-dihydrodiol generated by the action of dioxygenases at 9,10 and 7,8 positions, respectively in BaP [14, 18, 21]. Rentz et al. [21] demonstrated the presence of pyrene-7-hydroxy-8-carboxylic acid or pyrene-8-hydroxy-7-carboxylic acid which was hypothesized to be produced from transformation of 8-carboxy-7-hydroxypyrene. Nevertheless, in our study, presence of 8-carboxy-7-hydroxypyrene is detected, instead of pyrene-7-hydroxy-8-carboxylic

acid or pyrene-8-hydroxy-7-carboxylic acid. Formation of chrysene-4 or 5-carboxylic acid is presumed to be the end product of ortho cleavage ring fission product 4,5-chrysene dicarboxylic acid whose precursor is benzo(a)pyrene-cis-4,5- dihydrodiol produced by reaction of dioxygenation of BaP in the K-region (4,5 positions) as reported in *Mycobacterium vaanbellenii* PYR-1 [18].

To the best of our knowledge, the present study is the first one to report many metabolites of BaP degradation in genus *Bacillus*. In addition, a metabolic pathway of BaP has been elucidated in much greater detail for the first time in genus *Bacillus*. The results of this study on *B. subtilis* BMT4i (MTCC 9447), which is a potent BaP degrader (84.66% BaP degradation in 28 days) [19], could facilitate the elucidation of the biodegradation mechanism in *Bacillus*, which is currently not well explored. However, further studies are essential to find out whether the entire biodegradation process in *B. subtilis* results into the metabolic detoxification of BaP or not.

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