Missouri University of Science and Technology
Scholars' Mine

Chemistry Faculty Research & Creative Works

Chemistry

01 May 2022

Establishing Pteridine Metabolism in a Progressive Isogenic Breast Cancer Cell Model – Part II

Lindsey Rasmussen

Zachary Foulks

Jiandong Wu
Missouri University of Science and Technology, jwnc5@mst.edu

Casey Burton

et. al. For a complete list of authors, see https://scholarsmine.mst.edu/chem_facwork/3198

Follow this and additional works at: https://scholarsmine.mst.edu/chem_facwork

Part of the Biochemical and Biomolecular Engineering Commons, and the Chemistry Commons

Recommended Citation

L. Rasmussen et al., "Establishing Pteridine Metabolism in a Progressive Isogenic Breast Cancer Cell Model – Part II," *Metabolomics*, vol. 18, no. 5, article no. 27, Springer, May 2022.
The definitive version is available at https://doi.org/10.1007/s11306-022-01885-9

This Article - Journal is brought to you for free and open access by Scholars' Mine. It has been accepted for inclusion in Chemistry Faculty Research & Creative Works by an authorized administrator of Scholars' Mine. This work is protected by U. S. Copyright Law. Unauthorized use including reproduction for redistribution requires the permission of the copyright holder. For more information, please contact scholarsmine@mst.edu.

Metabolomics (2022) 18:27
https://doi.org/10.1007/s11306-022-01885-9

ORIGINAL ARTICLE

Establishing pteridine metabolism in a progressive isogenic breast cancer cell model – part II

Lindsey Rasmussen[1] · Zachary Foulks[1] · Jiandong Wu[2] · Casey Burton[1,3,4] · Honglan Shi[1,3]

Received: 22 February 2022 / Accepted: 1 April 2022 / Published online: 28 April 2022
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2022

Abstract

Introduction Determining the biological significance of pteridines in cancer development and progression remains an important step in understanding the altered levels of urinary pteridines seen in certain cancers. Our companion study revealed that several folate-derived pteridines and lumazines correlated with tumorigenicity in an isogenic, progressive breast cancer cell model, providing direct evidence for the tumorigenic origin of pteridines.

Objectives This study sought to elucidate the pteridine biosynthetic pathway in a progressive breast cancer model via direct pteridine dosing to determine how pteridine metabolism changes with tumorigenicity.

Methods First, MCF10AT breast cancer cells were dosed individually with 15 pteridines to determine which pteridines were being metabolized and what metabolic products were being produced. Second, pteridines that were significantly metabolized were dosed individually across the progressive breast cancer cell model (MCF10A, MCF10AT, and MCF10ACA1a) to determine the relationship between each metabolic reaction and breast cancer tumorigenicity.

Results Several pteridines were found to have altered metabolism in breast cancer cell lines, including pterin, isoxanthopterin, xanthopterin, sepiapterin, 6-biopterin, lumazine, and 7-hydroxylumazine ($p < 0.05$). In particular, isoxanthopterin and 6-biopterin concentrations were differentially expressed ($p < 0.05$) with respect to tumorigenicity following dosing with pterin and sepiapterin, respectively. Finally, the pteridine biosynthetic pathway in breast cancer cells was proposed based on these findings.

Conclusions This study, along with its companion study, demonstrates that pteridine metabolism becomes disrupted in breast cancer tumor cells. This work highlights several key metabolic reactions within the pteridine biosynthetic pathway that may be targeted for further investigation and clinical applications.

Keywords Pteridine · Breast cancer · Pteridine metabolism · MCF10A cell line · HPLC-MS/MS · Biomarker

1 Introduction

Pteridines are metabolic intermediates in the biosynthetic pathways of various cofactors and vitamins (Burton et al., 2016a; Crabtree et al., 2009; Fukushima & Shiota, 1974; Gross & Levi, 1992). Elevated levels of urinary pteridines have been reported in patients with certain cancers (Burton et al., 2013, 2016b; Gamagedara et al., 2011; Kośliński et al., 2011; Ma & Burton, 2013; Shantaram et al., 2012), prompting interest in elucidating the role of pteridines in cancer metabolism. However, previous efforts to study the potential tumorigenic origin of pteridines have been met with analytical challenges, including trace cellular concentrations, a multitude of pteridine derivatives with diverse chemical functionality, multiple structural isomers, and rapid autooxidation and degradation of certain pteridine derivatives (Burton & Ma, 2017, 2019; Kośliński et al., 2011; Ma & Burton, 2013). Recent advances in pteridine

✉ Casey Burton
c.f.burton@mst.edu

✉ Honglan Shi
honglan@mst.edu

1 Department of Chemistry, Missouri University of Science and Technology, 400 W 11th Street, 65409 Rolla, MO, USA

2 Department of Chemical and Biochemical Engineering, Missouri University of Science and Technology, 65409 Rolla, MO, USA

3 Center for Biomedical Research, Missouri University of Science and Technology, 65409 Rolla, MO, USA

4 Phelps Health, 65401 Rolla, MO, USA

quantification in relevant biological matrices have newly enabled comprehensive investigations into the pteridine biosynthetic pathway in cellular disease models (Burton et al., 2015).

To this end, our team recently explored how tumorigenicity affects pteridine metabolism using a novel isogenic, progressive breast cancer cell model consisting of sequentially derived cell lines that models the clinical progression towards metastatic breast cancer (Rasmussen et al., 2022). In our companion study, we reported that extracellular levels of pterin and 6-hydroxylumazine were correlated with tumorigenicity following dosing with folic acid, one of the two primary precursors to the pteridine biosynthetic pathway. This work provided direct evidence for the tumorigenic origin of elevated pteridines seen in urine, and particularly in breast cancer (Burton et al., 2014, 2016b; Gamagedara et al., 2011). However, the manner in which the pteridine biosynthetic pathway may differ in breast tumor cells and the molecular mechanisms responsible for these changes remain to be determined.

Moreover, several downstream pteridines were observed to have altered extracellular levels following folic acid dosing in our companion study, such as 6-carboxypterin, 6-hydroxymethylpterin, and xanthopterin, that were not otherwise correlated with tumorigenicity (Rasmussen et al., 2022). Furthermore, other downstream pteridines derived from folic acid were not otherwise detected following folic acid dosing in the companion study. For example, isoxanthopterin, which has been reported to be elevated in the urine of breast cancer patients (Burton et al., 2014, 2016b) and is the direct product of pterin (Blau et al., 1996; Fukushima & Shiota, 1974; Rembold et al., 1971). It is likely that dosing cells with folic acid, as a primary precursor to the pteridine biosynthetic pathway, may have been insufficient to produce detectable changes in secondary and tertiary metabolic products further downstream in the pathway. This study therefore sought to investigate the metabolism of downstream pteridines in a progressive breast cancer model by directly dosing cells with intermediate pteridines.

In addition, the companion study was limited in scope in that it strictly examined folate-derived pteridines, whereas previous work has demonstrated crosstalk between the folic acid and guanosine triphosphate (GTP) entry points to the pteridine biosynthetic pathway (Burton et al., 2016a; Oettle & Reibnegger, 1999; Rembold & Simmersbach, 1969). For example, urinary levels of 6-biopterin, xanthopterin, and neopterin were decreased in healthy individuals following a two-week folate supplementation (Burton et al., 2016a). Similarly, multiple pteridines throughout the pathway have been established as substrates for xanthine oxidoreductase (XOR) (Blau et al., 1996; Oettle & Reibnegger, 1999; Rembold et al., 1971) and pterin deaminase, which is responsible for the conversion of pteridines to lumazines (Fan et al., 2013; Jayaraman et al., 2016; Rembold & Simmersbach, 1969). The XOR enzyme has been shown to have altered activity in breast cancer cells (Battelli et al., 2015; Fini et al., 2008, 2011; Linder et al., 2005), which may explain the observations made in our companion study and provide suitable targets for additional investigation in this study. In this way, the current study further sought to investigate pteridine metabolism in a broader context that included key pteridines and lumazines derived from both GTP and folic acid. While previous studies have variously examined the clinical and biological significance of pteridines in breast cancer in urine (Burton et al., 2016a, b; Gamagedara et al., 2011) and cumulative effects of folate dosing in a progressive breast cancer cell model (Rasmussen et al., 2022), the elucidation of the pteridine biosynthetic pathway in progressive breast cancer has not yet been achieved. This study advanced this objective by sequentially dosing cells with pteridine intermediates using a progressive breast cancer cell model to determine pathway connectivity.

2 Experimental

2.1 Chemicals and materials

The MCF10A progressive breast cancer cell model, which consisted of three sequentially derived human epithelial breast cell lineages comprising nontumorigenic MCF10A (A), premalignant/tumorigenic MCF10AT (T), and tumorigenic/metastatic MCF10CA1a (C), was obtained from the Bargara Ann Karmanos Cancer Institute (Detroit, MI, USA) at Wayne State University, and matches the disease model used in our recent companion study (Rasmussen et al., 2022). Dithiothreitol, ammonium hydroxide, LC-MS grade formic acid, acetonitrile, methanol, and cell culture media were also all from the same resource of our recent study (Rasmussen et al., 2022). Ultrapure water was obtained from a Milli-Q Advantage® A10 and Millipore Elix® water purification system. All the pteridine standards, including leucopterin, were also purchased from Schircks Laboratory (Jona, Switzerland). The stock standard solutions of each pteridine were also prepared in the same way. Leucopterin was prepared at 1 mg/L in ultrapure water with 3.7% 2 N ammonium hydroxide measured quantitatively in this study owing to its poor solubility.

2.2 HPLC-MS/MS method

The HPLC-MS/MS method to determine pteridine derivatives was the same with our recent companion study (Rasmussen et al., 2022) with slight modifications, as described

in the supplemental information (**Tables S1** and **S2**). A procedural calibration method was used for HPLC-MS/MS analysis to minimize matrix effects and improve method accuracy. Briefly, different concentrations of pteridine standards were added in the cell culture media and processed through the same procedure as the sample preparation, and then diluted and analyzed as the same process of the sample analysis. Leucopterin was added to the method in a later phase of the study. It was only used for compound identification and confirmation purposes, not for quantitative analysis due to poor solubility of this standard.

2.3 Cell Culture, Pteridine Dosing, and Sample Preparation

Cells from each of the A, T, and C cell lines were cultivated in 100 mm cell culture dishes in 6 mL of cell culture media as described in our recent publication (Rasmussen et al., 2022) by following the protocol provided by the cell supplier. Pteridine dosing experiments were conducted using modified growth media containing 2 mg/L of the selected individual pteridine standard. In addition, two types of control samples, one consisting of dosed cell media without cells and the other one with cells but no pteridine dosing, were also processed in parallel for each batch of experiments. All the treatments were triplicated or duplicated. Cells and corresponding control samples were cultured for five days before harvesting. Cell media was harvested as 1 mL aliquots, and either used directly for extracellular pteridine analysis or stored at -80 °C until analysis, by following the same sample preparation and HPLC-MS/MS analysis method (Rasmussen et al., 2022). The cells were harvested and counted as described in our companion study (Rasmussen et al., 2022).

Two phases of experiments were performed in this study. First, T breast cancer cells were dosed individually with 15 pteridines. These step-by-step tests confirmed which pteridines were being metabolized and what metabolic products were being produced in each individual step of the metabolic pathway. Second, pteridines that were significantly metabolized from the phase 1 experiments were chosen to test in the phase 2 experiments by dosing each pteridine individually across all three progressive breast cancer cell lines (A, T, and C) to determine the relationship between each metabolic reaction and breast cancer tumorigenicity.

3 Results And Discussion

3.1 Dosing Study for metabolic pathway confirmation

3.1.1 Screening study for Pteridines metabolized by breast cells

Fifteen pteridines were dosed separately in breast cells (T cells) and downstream pteridines were monitored to determine pathway connectivity. Dosed concentrations of

Table 1 Production of cell-derived pteridines following dosing with different pteridine precursors. Samples were dosed for 5 days with each initial pteridine concentration at 2 mg/L. Reported concentrations have been normalized relative to pteridines measured in dosed media without cells

Dosed Pteridine* (Abbreviation)	µM	Amount Remaining (%)	Pteridines Produced (Abbreviation)	µM
Pterin (PTE)	12.3	3	Isoxanthopterin (ISO)	7.52
			7-Hydroxylumazine (7-HLUM)	0.108
			6-Hydroxylumazine (6-HLUM)	0.005
Xanthopterin (XAN)	11.2	5	7,8-Dihydroxanthopterin (XH_2)	0.157
Sepiapterin (SEP)	8.43	43	6-Biopterin (BIO)	0.299
			7,8-Dihydroxanthopterin (XH_2)	0.241
			7-Hydroxylumazine (7-HLUM)	0.141
			Isoxanthopterin (ISO)	0.091
			Xanthopterin (XAN)	0.069
			6-Hydroxylumazine (6-HLUM)	0.057
Lumazine (LUM)	12.2	0	7-Hydroxylumazine (7-HLUM)	7.34
6-Hydroxylumazine (6-HLUM)	11.1	38		
Undosed		N/A	6-Hydroxymethylpterin** (6-HMP)	0.169

*Additional pteridines that were dosed, but otherwise not significantly metabolized by cell samples, are not included in this table. They are 6-Methylpterin (6-MP), Isoxanthopterin, 7-Hydroxylumazine, 7,8-Dihydroxanthopterin, 6,7-Dimethylpterinn (6,7-DMP), 6-Hydroxymethylpterin, 6-Carboxypterin (6-CAP), 6-Biopterin, Neopterin (NEO), and Monapterin (MNP)

**6-Hydroxymethylpterin was detected in all samples at similar concentrations, including in control cells without pteridine dosing, therefore not considered as direct product of dosed pteridines

five pteridines, including pterin, xanthopterin, sepiapterin, lumazine, and 6-hydroxylumzine, were significantly reduced after five days (Table 1). 6-Hydroxymethylpterin was detected in all extracellular media of the cell samples in a concentration range of 0.08–0.17 µM, which is in agreement with Rasmussen et al., (2022) where 6-hydroxymethylpterin was detected in T cells at low concentrations without additional dosed folic acid.

The various pteridine products observed following dosing with the pteridine precursors, as shown in Table 1, align with the established pteridine biosynthetic pathway (Fig. 1) (Burton et al., 2016a; Crabtree et al., 2009; Kośliński et al., 2011; Rasmussen et al., 2022; Rembold et al., 1971). Several pteridine derivatives, including 6-hydroxymethylpterin, 7-hydroxylumazine, isoxanthopterin, 6-biopterin, 6-methylpterin, 6,7-dimethylpterin, neopterin, and monapterin, were not significantly metabolized by the breast cancer cells, indicating that these pteridine derivatives may be terminal endpoints within the pteridine biosynthetic pathway or that these pteridine derivatives may not be actively transported into breast cancer cells. Evidence for the latter may include 7,8-dihydroxanthopterin, a precursor to xanthopterin, and 6-carboxypterin, a precursor to pterin (Burton et al., 2016a; Rasmussen et al., 2022; Rembold et al., 1971), which were not shown to be significantly metabolized in these experiments. The detailed results from each dosing experiment are discussed below.

3.1.2 6-Carboxypterin dosing results

Pterin may be formed from folic acid either (1) nonenzymatically through the photocatalysis of folic acid or (2) through a multistep enzymatic process involving dihydrofolate reductase and XOR (Burton et al., 2016a; Rasmussen et al., 2022; Rembold et al., 1971).

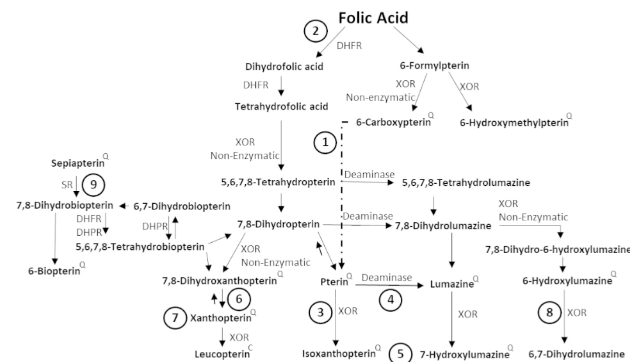


Fig. 1 Pteridine pathway in breast cells based on screening and quantitative studies. Circled numbers indicate pathways evaluated and discussed in this study. (Q) and (C) indicate compounds analyzed quantitatively and confirmatively, respectively, in the pteridine panel

$$\text{Folic Acid} \rightarrow\rightarrow \text{6-Carboxypterin} \rightarrow \text{Pterin} \quad \text{(pathway 1)}$$

$$\text{Folic Acid} \rightarrow \text{(DHFR)} \rightarrow\rightarrow \text{Pterin} \quad \text{(pathway 2)}$$

In this experiment, 6-carboxypterin was not significantly metabolized by the premalignant T cell line and no additional pterin was observed following dosing with 6-carboxypterin. This finding generally agreed with that of our companion study where folic acid dosing yielded substantial amounts of 6-carboxypterin and only 10–35 µg/L of pterin (Rasmussen et al., 2022). Both studies were performed under dark conditions to minimize the photocatalytic degradation of folic acid to 6-carboxypterin and ultimately pterin (Rasmussen et al., 2022). These findings together suggest that the metabolic pathway from folic acid to pterin in breast cancer cells proceeds primarily through enzymatic processes involving dihydrofolic acid as an intermediate (pathway 2) and not through physical processes involving 6-carboxypterin as an intermediate (pathway 1). This understanding would further explain the minimal change in urinary pterin following folate supplementation in healthy individuals reported by Burton et al., (2016a).

3.1.3 Pterin derived metabolic pathway

Pterin dosing. In this experiment, pterin dosing afforded isoxanthopterin along with trivial levels of 6-hydroxylumazine and 7-hydroxylumazine. The conversion of pterin to isoxanthopterin has been previously established as an enzymatic reaction involving XOR both clinically in humans (Blau et al., 1996) and *in vitro* using XOR enzyme derived from rat liver (Rembold et al., 1971). This experiment confirmed this reaction step (pathway 3) by directly dosing pterin precursor into the breast cancer cell culture.

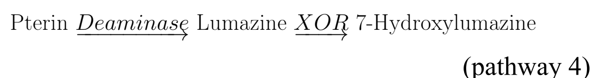
$$\text{Pterin} \xrightarrow{XOR} \text{Isoxanthopterin} \quad \text{(pathway 3)}$$

In our companion study, isoxanthopterin was not detected at significant levels following folate dosing; however, the amount of pterin precursor generated with folic acid dosing in that study was approximately 10–35 µg/L whereas the pterin precursor dose used in the current study was 2 mg/L. In this way, the negligible effects of daily folate supplementation for two weeks on urinary isoxanthopterin (Burton et al., 2016a) are likely the result of the poor conversion efficiency of folic acid to pterin.

Pterin dosing yielded a small fraction (0.08%) of 6-hydroxylumazine. This unexpected finding may suggest

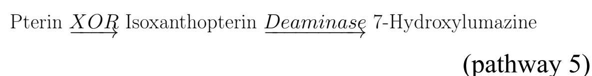
a reverse equilibrium between pterin and 7,8-dihydropterin. The latter may be deaminated to 7,8-dihydrolumazine and ultimately to 6-hydroxylumazine through previously established reactions (Rembold et al., 1971). However, 7,8-dihydropterin was not monitored in this study due to lack of chemical standard, and its reaction rate constant and whether it is enzymatically controlled has not been previously reported. We also dosed 6-hydroxylumazine directly in the cell culture, and the 6-hydroxylumazine was significantly metabolized by the T cells with only 38% of the dosed amount remaining after five days (Table 1). However, no metabolic products were detected in our pteridine panel, which excluded 6,7-dihydroxylumazine, the predicted metabolic product (Fig. 1, pathway 8) (Rembold et al., 1971) due to a lack of commercially available standard. This rapid conversion of 6-hydroxylumazine to the downstream product(s) may explain its low yield with pterin dosing.

Lumazine dosing. Lumazine strongly converted to 7-hydroxylumazine (pathway 4), with a molar conversion rate of 60%, agreeing with a previous study that 7-hydroxylumazine is the direct product of lumazine (Rembold et al., 1971). In addition, while lumazine was not detected in the T cell line when dosed with pterin, subsequent pterin dosing in the highly tumorigenic C cell line yielded lumazine (pathway 4), which is discussed in Sect. 3.2.1.



(pathway 4)

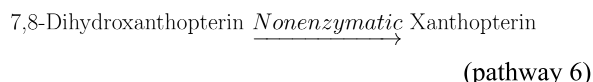
Isoxanthopterin dosing. Isoxanthopterin was not metabolized in the cell samples in the screening experiment. In a prior work by Rembold et al. (1969), a pterin deaminase was shown *in vitro* to deaminate 5,6,7,8-tetrahydropterin, 7,8-dihydropterin, pterin, and isoxanthopterin to 5,6,7,8-tetrahydrolumazine, 7,8-dihydrolumazine, lumazine, and 7-hydroxylumazine, respectively. However, in this experiment, dosed isoxanthopterin was not metabolized by the cells, indicating that the hypothesized deaminase responsible for generating the lumazines in the breast cells does not act on isoxanthopterin. Therefore, it is proposed that the generation of 7-hydroxylumazine in breast cells dosed with pterin is due to pterin conversion to lumazine (pathway 4) and not from deamination of isoxanthopterin (pathway 5).



(pathway 5)

3.1.4 7,8-Dihydroxanthopterin and xanthopterin dosing results

7,8-Dihydroxanthopterin dosing. Dosed 7,8-dihydroxanthopterin was not significantly metabolized by the T cell line. Instead, similar ratios of 7,8-dihydroxanthopterin and xanthopterin were observed in dosed cells and media (2:1 and 2.6:1, respectively). This may be attributed to the spontaneous autooxidation of 7,8-dihydroxanthopterin to xanthopterin (Blau et al., 1996; Burton et al., 2016a; Rembold et al., 1971).



(pathway 6)

Xanthopterin dosing. Cells dosed with xanthopterin resulted in qualitatively higher levels of leucopterin (**Fig. S1**). This finding agreed with previously reported results that leucopterin is a metabolic product of xanthopterin when its metabolism was studied *in vitro* using XOR enzyme derived from rat liver (Rembold et al., 1971) and butterflies (Watt, 1972). Xanthopterin dosing also yielded marginal amounts (1.41%) of 7,8-dihydroxanthopterin. This finding suggested that while 7,8-dihydroxanthopterin incompletely oxidizes to xanthopterin, as already seen here, that some 7,8-dihydroxanthopterin may be recovered from as yet undetermined processes (pathway 7).

(pathway 7)

3.1.5 Sepiapterin dosing results

The sepiapterin dosing experiment resulted in significant increases of multiple pteridine derivatives, including 6-biopterin, 7,8-dihydroxanthopterin, 7-hydroxylumazine, isoxanthopterin, xanthopterin, and 6-hydroxylumazine (Table 1). The multiplicity of metabolic products seen here can be attributed to the so-called salvage pathway in which exogenous sepiapterin is reduced to 7,8-dihydrobiopterin by sepiapterin reductase (Fig. 1, pathway 9) and subsequently reduced to 5,6,7,8-tetrahydrobiopterin by dihydrofolate reductase (Alp & Channon, 2004; Crabtree et al., 2009; Nichol et al., 1983; Tomšíková et al., 2013). A two-step spontaneous oxidation of 5,6,7,8-tetrahydrobiopterin with 7,8-dihydrobiopterin as an intermediate yields 6-biopterin. Moreover, nonenzymatic side chain cleavage of 5,6,7,8-tetrahydrobiopterin can yield 7,8-dihydropterin (Walter et al., 1997). The latter may be spontaneously oxidized to pterin

and subsequently converted to isoxanthopterin by XOR, as shown in this study and others (Rembold et al., 1971; Tomšíková et al., 2013; Walter et al., 1997). The intermediate 7,8-dihydropterin may also be converted to 7,8-dihydroxanthopterin by XOR (Rembold et al., 1971). Similarly, 6-hydroxylumazine and 7-hydroxylumazine may be produced from 7,8-dihydropterin and pterin, as seen in the current study and elsewhere (Rembold et al., 1971).

In this way, a full accounting of the observed metabolic products of sepiapterin may be understood through its salvage to 5,6,7,8-tetrahydrobiopterin. Given the clinical significance of many of these pteridine derivatives in various epithelial cancers, this finding may be particularly important in understanding their biological significance in cancer development and the potential role of 5,6,7,8-tetrahydrobiopterin metabolism in particular.

3.2 Effects of Tumorigenicity on Pteridine Metabolisms

While the screening study in phase 1 was designed to determine the metabolic products of various pteridine precursors within the T cell line, the quantitative study in phase 2 was designed to determine the relationship between individual metabolic reactions and breast cancer tumorigenicity across the progressive breast cancer cell model. For these experiments, three exogeneous pteridine precursors, including pterin, xanthopterin, and sepiapterin, were dosed in the A, T, and C cell lines and the amounts of precursor consumed and products formed were quantified. These pteridine precursors were selected based on the multiplicity of products covered, their clinical significance as potential cancer biomarkers, and to better elucidate their biosynthetic pathways in breast tumors.

3.2.1 Pterin precursor

Exogeneous pterin was efficiently converted to isoxanthopterin with an 86.26% and 88.44% molar conversion in the nontumorigenic A cell line and premalignant T cell lines, respectively. Less conversion was observed (66%) in the tumorigenic C cell line (**Table S3**). When normalized to cell concentrations (Fig. 2), isoxanthopterin was marginally higher in T and C cell lines ($p < 0.05$, Student's t-test), 6-hydroxymethylpterin and lumazine were significantly higher in C cell lines ($p < 0.05$, Student's t-test), and 6-hydroxylumazine generally trended with tumorigenicity although no pairwise comparisons were statistically significant. Notably, the amount of unmetabolized pterin in C cells after five days was significantly higher than in A and T cells ($p < 0.05$, Student's t-test). This finding, taken together with the elevated levels of pterin in tumorigenic breast cancer cells following folic acid dosing reported in our companion study (Rasmussen et al., 2022), strongly implicates altered XOR enzymatic activity. As previously noted, XOR mediates a myriad of pteridine and lumazine reactions, including the conversion of pterin to isoxanthopterin. While XOR activity was not directly measured in the present study, its activity has previously been shown to be inversely correlated with breast cancer tumorigenicity using the same progressive breast cancer cell model (Fini et al., 2008, 2011). In this case, suppressed enzymatic activity in tumor cells would be expected to result in less efficient conversion of pterin, as seen here and in our companion

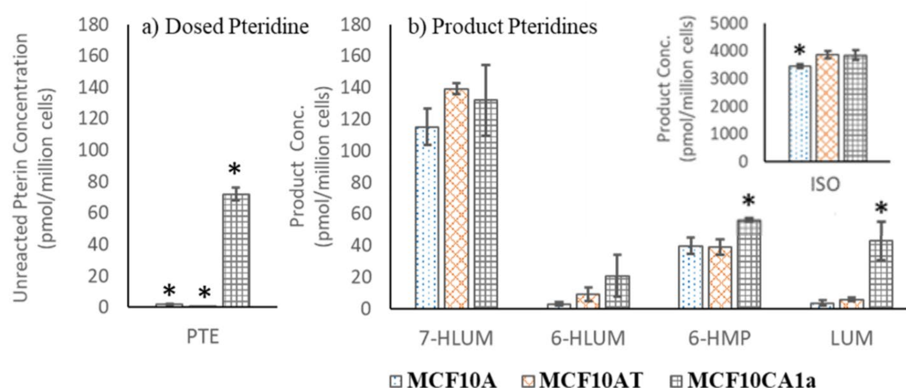


Fig. 2 Production of pteridine derivatives in different breast cells following dosing with PTE (12.26 µM) for five days (n = 3). (a) Concentration (pmol/million cells) of unreacted PTE in different breast cells; (b) Concentrations (pmol/million cells) of pteridine derivatives produced following dosing with PTE in different breast cells; (c) molar reaction recoveries following PTE dosing * $p < 0.05$, two-tailed Student's t-test

c)

Molar Reaction Recoveries	MCF10A	MCF10AT	MCF10CA1a
ISO produced	86.26%	88.44%	66.09%
Other pteridines produced	< 5%	< 5%	< 5%
Total recovery	**89.34%**	**91.98%**	**70.59%**

study (Rasmussen et al., 2022). In addition, the tumorigenic increase of lumazine and, to a lesser extent, 6-hydroxylumazine, but not 7-hydroxylumazine, is suggestive of tumorigenic deaminase activity. As previously discussed, pterin may be deaminated to lumazine and subsequently hydroxylated to 7-hydroxylumazine by XOR (Fig. 1). In this way, suppressed XOR activity and elevated deaminase activity in tumorigenic breast cells would promote pterin conversion to lumazine, but hinder the subsequent reaction, resulting in higher levels of the lumazine intermediate. Similarly, pterin may be reduced to 7,8-dihydropterin, whereupon it may be deaminated and converted to 6-hydroxylumazine before being ultimately converted to 6,7-dihydroxylumazine by XOR (Fig. 1). In this way, the combination of increased deaminase activity and suppressed XOR activity can account for the majority of the metabolic products seen here. Finally, the marginal increases observed for isoxanthopterin and 6-hydroxymethylpterin in tumorigenic C cells suggest more complicated pathways, potentially involving novel feedback mechanisms and metabolic reactions. Taken together, these findings present an accounting of altered pterin metabolism in breast cancer, invoking multiple enzymatic processes and providing a tumorigenic origin for several pteridine derivatives.

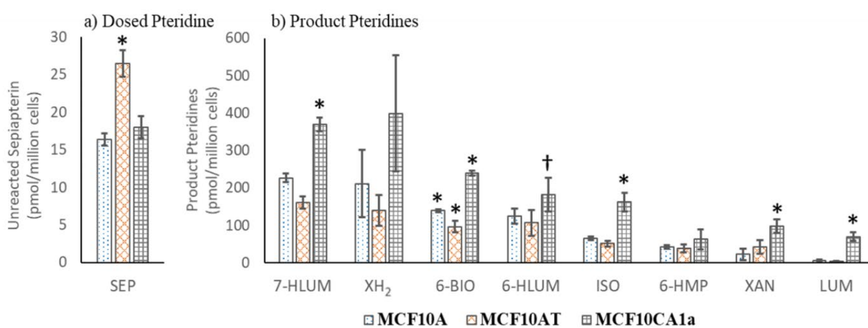
3.2.2 Xanthopterin precursor

More than 95% of the exogeneous xanthopterin precursor was metabolized by the A and T cell lines, while 77% was consumed by the tumorigenic C cell line (Table 2). The difference between the A and T cell lines and the C cell line was statistically significant ($p < 0.05$, Student's t-test). Notably, most of the consumed xanthopterin could not be accounted for by the monitored pteridine derivatives. Previous work has identified leucopterin as a primary metabolic product of xanthopterin (Rembold et al., 1971; Watt, 1972). Although leucopterin was not quantitatively determined in the current study, it was produced in this dosing experiment and confirmed by HPLC-MS/MS method matching elution times and ionization ion pairs with leucopterin standard, as shown

Table 2 Concentrations (pmol/million cells) of unreacted xanthopterin precursor and 7,8-dihydroxanthopterin product. Total reaction recovery (% molar concentration) of detected pteridines to initial xanthopterin dose (11.165 µM) for 5 days (n = 3). Standard deviations are denoted in parentheses

	Pteridine	MCF10A	MCF10AT	MCF10CA1a
Unreacted Precursor (pmol/million cells)	XAN	104.30 (7.86)	73.63 (17.62)	1391.75 (126.62)
Detected Product (pmol/million cells)	XH_2	179.02 (17.82)	65.54 (63.70)	112.75 (95.09)
Reaction Recovery (%)				
Unreacted Precursor	XAN	3.66 (0.28)	1.46 (0.35)	23.14 (2.11)
Detected Product	XH_2	6.29 (0.63)	1.30 (1.26)	1.91 (1.58)
	Sum	9.95 (0.90)	2.75 (1.61)	25.05 (3.69)
	LEU*	Detected	Detected	Detected

*Leucopterin confirmed, but not quantified



c)

Molar Reaction Recoveries	MCF10A	MCF10AT	MCF10CA1a
7-HLUM produced	10.32%	7.70%	9.34%
XH_2 produced	9.65%	6.63%	10.06%
6-BIO produced	6.36%	4.60%	6.04%
6-HLUM produced	5.68%	5.09%	4.59%
Other pteridines produced	< 5%	< 5%	< 5%
Total recovery	**36.94%**	**29.89%**	**38.77%**

Fig. 3 Production of pteridine derivatives in different breast cells following dosing with SEP (8.43 µM) for five days (n = 3). (a) Concentration (pmol/million cells) of unreacted SEP in different breast cells; (b) Concentrations (pmol/million cells) of pteridine derivatives produced following dosing with SEP in different breast cells; (c) molar reaction recoveries following SEP dosing * $p < 0.05$, two-tailed Student's t-test † Only the T and C cell lines were significantly different, $p < 0.05$, two-tailed Student's t-test

in the chromatograms in Fig. S1. Furthermore, the observed decrease in xanthopterin consumption in the C cell line provides indirect evidence for the conversion of the exogeneous xanthopterin to leucopterin, which is mediated by XOR. In this way, suppressed XOR activity in the tumorigenic C cell line would limit conversion of the exogeneous xanthopterin to leucopterin, as observed here. These findings ultimately suggest that xanthopterin, and potentially leucopterin, may exhibit altered metabolism in breast tumors.

3.2.3 Sepiapterin precursor

Exogeneous sepiapterin was virtually consumed (<2% remaining) across all three cell lines (**Table S4**). The amount of unmetabolized sepiapterin remaining was significantly higher in the T cell line ($p < 0.05$, Student's t-test). Exogeneous sepiapterin yielded a multiplicity of metabolic products, including 6-biopterin, 7,8-dihydroxanthopterin, isoxanthopterin, 6-hydroxylumazine, 7-hydroxylumazine, lumazine, and xanthopterin. Five of these metabolic products, shown in Fig. 3, were found to have significantly altered levels in the tumorigenic C cell line ($p < 0.05$, Student's t-test). Interestingly, pterin was not generated at detectable levels, although it is likely that the levels were below detection limits and that it was readily converted to isoxanthopterin, lumazine, and 7-hydroxylumazine. 6-Hydroxylumazine was only statistically discriminated between the T and C cell lines ($p < 0.05$) and non-statistically discriminated between the A and C cell lines ($p = 0.065$) while in our previous study, all three cell lines were correlated with tumorigenicity. However, there are several more metabolic steps between sepiapterin and 6-hydroxylumzine than from folic acid, as well as derivation from a GTP-derived precursor, which may indicate that biomarker levels may be affected by available precursors, which supports previous report from Burton et al., (2016a) wherein increasing available folate levels altered urinary pteridine levels.

Although this study was not designed to assess the individual intermediates within this section of the pteridine pathway, there are several enzymes implicated in this pathway, including XOR, SR, DHFR, DHPR, and pterin deaminase that may be useful targets for further investigation. Similarly, expansion of the pteridine panel to include reduced and semi-reduced pteridine derivatives may shed additional insights into the intermediate pathways of both pteridines and lumazines. Pterin deaminase, hypothesized to be responsible for the conversion of pteridines to lumazines. In this way, further work on elucidating the molecular mechanisms for the differentiation of these metabolic products and their corresponding enzymes in breast tumor cells may provide useful insights into the role of pteridines as breast cancer biomarkers.

4 Conclusions

In this study, the pteridine biosynthetic pathway in progressive breast cancer was investigated by dosing cells directly with pteridine intermediates and monitoring the dosed precursors and the metabolic products. Five pteridines, including pterin, xanthopterin, sepiapterin, lumazine, and 6-hydroxylumazine were found to be metabolized by breast cancer cells. Further investigation into the metabolism of pterin, xanthopterin, and sepiapterin revealed differential patterns of pteridine metabolites as a function of breast cancer tumorigenicity. Specifically, several pteridines, including pterin, isoxanthopterin, xanthopterin, sepiapterin, 6-biopterin, lumazine, and 7-hydroxylumazine were reported to have significantly altered concentrations ($p < 0.05$) across the progressive breast cancer model. Pteridine concentrations were found to generally peak in the most aggressive MCF10CA1a cell line, although, sepiapterin, 6-biopterin, and isoxanthopterin were also significantly altered in the mildly aggressive MCF10AT cell line. In this way, the present work built upon our recent companion study to provide direct evidence for the tumorigenic origin of pteridines in breast cancer. These findings together provide an important biological basis for the accounts of elevated urinary pteridines reported in women with breast cancer. Overall, the current study provided new insights into the pteridine biosynthetic pathway and its role in breast cancer development and progression, identifying multiple pteridine metabolites and reactions that warrant further investigation as clinical biomarkers.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s11306-022-01885-9.

Acknowledgements The authors would like to thank Barbara Ann Karmanos Cancer Institute for providing the MCA 10 A series of cell lines. We appreciate the assistances of Dr. Paul Nam on analytical method and instrument troubleshooting. This study was funded by National Institute of Health (NIH), National Cancer Institute, award number R03CA219337.

Author contribution Statement All authors contributed to the study conception and design and all authors commented on previous versions of the manuscript. LR, ZF, and JW conducted experiments and wrote the first draft of the manuscript. CB and HS conceived and designed research, analyzed data, and they also edited this manuscript. All authors read and approved the final manuscript.

Funding This study was funded by National Institute of Health (NIH), National Cancer Institute, award number R03CA219337.

Availability of data and material The data used in this study are available from the corresponding author upon reasonable request.

Code Availability Not applicable.

Declarations

Conflict of interest All the author declare that they have no conflict of interest.

Compliance with ethical standards This article does not contain any studies with human and/or animal participants performed by any of the authors.

Cell lines The cell lines, including MCF10A, MCF10AT, and MCF-10CA1a.cl1, was provided in October, 2019 by Barbara Ann Karmanos Cancer Institute (4100 John R, Detroit, Michigan 48201) at Wayne State University with a non-exclusive license agreement.

Ethics approval Not applicable.

Consent to participate Not applicable.

References

Alp, N. J., & Channon, K. M. (2004). Regulation of Endothelial Nitric Oxide Synthase by Tetrahydrobiopterin in Vascular Disease. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 24, 413–420. https://doi.org/10.1161/01.atv.0000110785.96039.f6

Battelli, M. G., Polito, L., Bortolotti, M., & Bolognesi, A. (2015). Xanthine oxidoreductase in cancer: more than a differentiation marker. *Cancer Medicine*, 5, 546–557. https://doi.org/10.1002/cam4.601

Blau, N., De Klerk, J. B. C., Thöny, B., Heizmann, C. W., Kierat, L., Smeitink, J. A. M., & Duran, M. (1996). Tetrahydrobiopterin Loading Test in Xanthine Dehydrogenase and Molybdenum Cofactor Deficiencies. *Biochemical and Molecular Medicine*, 58, 199–203. https://doi.org/10.1006/bmme.1996.0049

Burton, C., Shi, H., & Ma, Y. (2013). Simultaneous Detection of Six Urinary Pteridines and Creatinine by High-Performance Liquid Chromatography-Tandem Mass Spectrometry for Clinical Breast Cancer Detection. *Analytical Chemistry*, 85, 11137–11145. https://doi.org/10.1021/ac403124a

Burton, C., Shi, H., & Ma, Y. (2014). Normalization of urinary pteridines by urine specific gravity for early cancer detection. *Clinica Chimica Acta*, 435, 42–47. https://doi.org/10.1016/j.cca.2014.04.022

Burton, C., Weng, R., Yang, L., Bai, Y., Liu, H., & Ma, Y. (2015). High-throughput intracellular pteridinic profiling by liquid chromatography–quadrupole time-of-flight mass spectrometry. *Analytica Chimica Acta*, 853, 442–450. https://doi.org/10.1016/j.aca.2014.10.044

Burton, C., Shi, H., & Ma, Y. (2016a). Daily variation and effect of dietary folate on urinary pteridines. *Metabolomics*, 12, 1–10. https://doi.org/10.1007/s11306-016-1019-4

Burton, C., Shi, H., & Ma, Y. (2016b). Development of a high-performance liquid chromatography – Tandem mass spectrometry urinary pterinomics workflow. *Analytica Chimica Acta*, 927, 72–81. https://doi.org/10.1016/j.aca.2016.05.005

Burton, C., & Ma, Y. (2017). The role of urinary pteridines as disease biomarkers. *Pteridines*, 28, 1–21. https://doi.org/10.1515/pterid-2016-0013

Burton, C., & Ma, Y. (2019). Current Trends in Cancer Biomarker Discovery Using Urinary Metabolomics: Achievements and New Challenges. *Current Medicinal Chemistry*, 26, 5–28. https://doi.org/10.2174/0929867324666170914102236

Crabtree, M. J., Tatham, A. L., Hale, A. B., Alp, N. J., & Channon, K. M. (2009). Critical Role for Tetrahydrobiopterin Recycling by Dihydrofolate Reductase in Regulation of Endothelial Nitric-oxide Synthase Coupling. *The Journal of Biological Chemistry*, 284, 28128–28136. https://doi.org/10.1074/jbc.M109.041483

Fan, H., Hitchcock, D. S., Seidel, R. D., Hillerich, B., Lin, H., Almo, S. C. … Raushel, F. M. (2013). Assignment of Pterin Deaminase Activity to an Enzyme of Unknown Function Guided by Homology Modeling and Docking. *Journal of the American Chemical Society*, 135, 795–803. https://doi.org/10.1021/ja309680b

Fini, M. A., Orchard-Webb, D., Kosmider, B., Amon, J. D., Kelland, R., Shibao, G., & Wright, R. M. (2008). Migratory activity of human breast cancer cells is modulated by differential expression of xanthine oxidoreductase. *Journal of Cellular Biochemistry*, 105, 1008–1026. https://doi.org/10.1002/jcb.21901

Fini, M. A., Monks, J., Farabaugh, S. M., & Wright, R. M. (2011). Contribution of Xanthine Oxidoreductase to Mammary Epithelial and Breast Cancer Cell Differentiation In Part Modulates Inhibitor of Differentiation-1. *Molecular Cancer Research*, 9, 1242–1254. https://doi.org/10.1158/1541-7786.MCR-11-0176

Fukushima, T., & Shiota, T. (1974). Biosynthesis of Biopterin by Chinese Hamster Ovary (CHO K1) Cell Culture. *Journal of Biological Chemistry*, 249, 4445–4451

Gamagedara, S., Gibbons, S., & Ma, Y. (2011). Investigation of urinary pteridine levels as potential biomarkers for noninvasive diagnosis of cancer. *Clinica Chimica Acta*, 412, 120–128. https://doi.org/10.1016/j.cca.2010.09.015

Gross, S. S., & Levi, R. (1992). Tetrahydrobiopterin synthesis. An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. *Journal of Biological Chemistry*, 267, 25722–25729

Jayaraman, A., Thandeeswaran, M., Priyadarsini, U., Sabarathinam, S., Nawaz, K. A. A., & Palaniswamy, M. (2016). Characterization of unexplored amidohydrolase enzyme—pterin deaminase. *Applied Microbiology and Biotechnology*, 100, 4779–4789. https://doi.org/10.1007/s00253-016-7513-9

Koślinski, P., Bujak, R., Daghir, E., & Markuszewski, M. J. (2011). Metabolic profiling of pteridines for determination of potential biomarkers in cancer diseases. *ELECTROPHORESIS*, 32, 2044–2054. https://doi.org/10.1002/elps.201000664

Linder, N., Lundin, J., Isola, J., Lundin, M., Raivio, K. O., & Joensuu, H. (2005). Down-Regulated Xanthine Oxidoreductase Is a Feature of Aggressive Breast Cancer. *Clinical Cancer Research*, 11, 4372–4381. https://doi.org/10.1158/1078-0432.ccr-04-2280

Ma, Y., & Burton, C. (2013). Pteridine detection in urine: the future of cancer diagnostics? *Biomarkers in Medicine*, 7, 679–681. https://doi.org/10.2217/bmm.13.88

Nichol, C. A., Lee, C. L., Edelstein, M. P., Chao, J. Y., & Duch, D. S. (1983). Biosynthesis of tetrahydrobiopterin by de novo and salvage pathways in adrenal medulla extracts, mammalian cell cultures, and rat brain in vivo. *Proceedings of the National Academy of Sciences* **80**, 1546–1550. https://doi.org/10.1073/pnas.80.6.1546

Oettle, K., & Reibnegger, G. (1999). Pteridines as inhibitors of xanthine oxidase: structural requirements. *Biochimica et Biophysica Acta*, 1430, 387–395

Rasmussen, L., Foulks, Z., Burton, C., & Shi, H. (2022). Establishing pteridine metabolism in a progressive isogenic breast cancer cell model. *Metabolomics*, 18, https://doi.org/10.1007/s11306-021-01861-9

Rembold, H., & Simmersbach, F. (1969). Catabolism of pteridine cofactors II. A specific pterin deaminase in rat liver. *Biochimica et Biophysica Acta*, 184, 589–596

Rembold, H., Metzger, H., & Gutensohn, W. (1971). Catabolism of Pteridine Cofactors: III. On the introduction of an oxygen function into position 6 of the pteridine ring. *Biochimica et Biophysica Acta*, 230, 117–126. https://doi.org/10.1016/0304-4165(71)90059-6

Shantaram, M., Bhaktha, G., Kumta, S., & Aziz, M. A. (2012). Evaluation of Urinary 6-Hydroxymethylpterin in Malignancies. *International Journal of Analytical, Pharmaceutical, and Biomedical Sciences*, 1, 71–74

Tomšíková, H., Tomšík, P., Solich, P., & Nováková, L. (2013). Determination of pteridines in biological samples with an emphasis on their stability. *Bioanalysis*, 5, 2307–2326. https://doi.org/10.4155/bio.13.194

Walter, R., Blau, N., Schaffner, A., Schneemann, M., Speich, R., Stocker, R. … Schoedon, G. (1997). Inhalation of the Nitric Oxide Synthase Cofactor Tetrahydrobiopterin in Healthy Volunteers. *American Journal of Respiratory and Critical Care Medicine*, 156, 2006–2010. https://doi.org/10.1164/ajrccm.156.6.96-12084

Watt, W. B. (1972). Xanthine Dehydrogenase and Pteridine Metabolism in *Colias* Butterflies. *Journal of Biological Chemistry*, 247, 1445–1451. https://doi.org/10.1016/S0021-9258(19)45578-6

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.