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Breath tests to phenotype drug disposition in oncology

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ABSTRACT

Breath tests have been investigated as diagnostic tools to phenotype drug disposition in cancer patients in the pursuit to individualize drug treatment in cancer.

The choice of the right phenotype probe is crucial and depends on the metabolic pathway of the anticancer agent of interest. Breath tests using orally or intravenously administered selective non-radioactive ^{13}C-labeled probes to noninvasively evaluate dihydropyrimidine dehydrogenase, CYP3A4 and CYP2D6 enzyme activity have been published.

Clinically, ^{13}C-dextromethorphan breath test to predict endoxifen levels in breast cancer patients and ^{13}C-uracil breath test to predict fluoropyrimidine toxicity in colorectal cancer patients are most promising. However, clinical benefit and cost-effectiveness of these phenotype breath tests need to be determined in order to make the transition from experimental setting to clinical practice as companion diagnostic tests.

INTRODUCTION

Since the discovery of the presence of more than 250 compounds in exhaled breath by Linus Pauling in 1971 [1], there is renewed interest in breath testing for use in medical practice [2;3]. Analysis of breath is commercially available, to detect organ rejection in heart transplant patients, to diagnose Helicobacter pylori infection, and to monitor breath gases in asthma and during anesthesia [4]. In oncology, with new techniques, breath volatile organic compounds (VOCs) may distinguish lung cancer patients from healthy individuals with a high degree of sensitivity and specificity [5].

Besides diagnosis of cancer, breath tests (BT) have been introduced in clinical practice to phenotype drug disposition. Variation in drug disposition is thought to be an important determinant of interpatient variability in efficacy and toxicity of anticancer agents. Genetics, physiology, diet, co-medications and environment are all critical factors contributing to this variation in drug response [6]. Pharmacogenetic tests are now available for a number of polymorphic enzymes (CYP2C9, CYP2C19 and CYP2D6). However, predicting phenotype from genotype is at best an estimate. Phenotype changes throughout life mainly due to drug-drug interactions and other epigenetic factors while genotype remains the same.

Phenotyping drug metabolizing enzymes and drug transporters may be used to individualize drug therapy [7]. Phenotype tests can be carried out using a drug probe at subtherapeutic doses, single or as part of a phenotyping cocktail [8] and evaluating the concentrations of either the probe or its metabolites in plasma [9], saliva [10], or urine [11]. However, the analysis of these samples requires, labor-intensive and time consuming LC-MS analysis. Finally, for each new specific probe a new assay should be developed and validated. BT provide a rapid (less than 1 hour), noninvasive alternative to phenotype drug disposition [12]. In this article we present an overview of BT developed to phenotype disposition of anticancer agents.

1. THE PRINCIPLE OF BTs TO PHENOTYPE DRUG DIS-POSITION

The clearance of endogenous substrates and exogenous probes may be used to study specific metabolic pathways or drug transporters to predict disposition and treatment outcome of anticancer agents.

By using labeled (e.g. [13]C) probes in phenotyping BTs, the amount of exhaled labeled probe metabolite (e.g. [13]CO$_2$) reflects the clearance of the probe by a specific metabolic route. For

example, ^{13}C-dextromethorphan (^{13}C-DM) has been used as a specific CYP2D6 phenotype probe [13]. ^{13}C-Dextromethorphan breath test (DM-BT) is dependent on CYP2D6 mediated O-demethylation which results in generation of $^{13}CO_2$ which is measured in expired breath over time (Figure 3.1). BTs are usually performed by administering a probe to subjects who have been fasting overnight to facilitate rapid gastrointestinal absorption and transport to the liver. Patients with severe lung, renal or hepatic impairment are considered not eligible to participate in phenotyping BTs. Patients should abstain from alcohol for at least 24 hours before the BT. Breath samples are collected just before and at single or various time points after probe administration. Whereas radio-active ^{14}C isotopes were used in the past, stable, non-radioactive ^{13}C labeled isotopes are routinely used nowadays for labeling probes [14].

$$R\text{-}O^{13}CH_3 \xrightarrow{[O]} H^{13}CHO \xrightarrow{[O]} H^{13}COOH \xrightarrow{[O]} H_2O \; + \; ^{13}CO_2$$
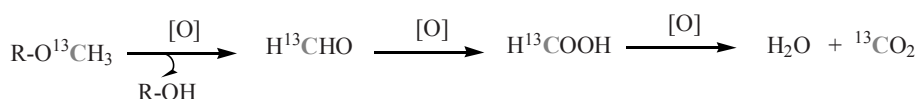$$\searrow R\text{-}OH$$

Figure 3.1   The principle of specific CYP2D6 phenotyping ^{13}C-dextromethorphan breath test (DM-BT), which is dependent on CYP2D6 *O*-demethylation. The released methyl group is involved in the formation of $^{13}CO_2$ that is released in expired breath over time.

2. ERYTHROMYCIN BREATH TEST

Introduction

In 1989, Watkins introduced the erythromycin breath test (ERMBT) to phenotype hepatic CYP3A [15]. After intravenous bolus injection of ^{14}C-labeled erythromycin it is N-demethylated by CYP3A leading to $^{14}CO_2$ in expired air which is trapped in a CO_2 binding solution at timed intervals. The rate of production of ^{14}C can then be calculated at the time of each collection based on an estimation of endogenous CO_2 generation [15;16].

In vitro correlation

Significant correlation was demonstrated between the ERMBT and *in vitro* erythromycin demethylation [17]. ERMBT results correlated well with hepatic microsomal CYP3A content in patients with severe liver disease [18] and in patients early after liver transplantation [17]. However, ERMBT results may be confounded by the additional affinity of erythromycin for P-glycoprotein [19;20] , solute carriers like OATP1A2 and OATP1B3 [21] and efflux transporters like ABCC2 [22]. ERMBT is further limited by the fact that erythromycin is a substrate for CYP3A4,

and not for CYP3A5 [23;24], which has a variable expression in patients and contributes to total CYP3A activity.

In vivo correlation with CYP3A substrates

In 16 cancer patients no significant correlation between ERMBT parameters and erythromycin plasma clearance was reported [25], which might be due to intravenous administration of ^{14}C-labeled erythromycin and therefore restriction of ERMBT to predict hepatic and not intestinal CYP3A4 phenotype.

ERMBT parameters were found to correlate with known substrates of CYP3A4, such as cyclosporine [26-28]. For midazolam, results were conflicting: Lown found a significant correlation between ERMBT and intravenous midazolam clearance [29], where others [21;30;31] reported no correlation. In 14 young healthy white men, no significant correlation was demonstrated between metabolism of intravenously administered CYP3A4 substrate alfentanil and ERMBT [32]. Subjects who took the CYP3A inhibitors triacetyloleandomycin or ketoconazole, ERMBT results were significantly reduced [33]. In contrast, the strong CYP3A inducer rifampicin, increased ERMBT values dramatically [15;34].

Correlation with pharmacokinetics of anticancer agents

Pharmacokinetics of CYP3A metabolized anticancer agents docetaxel, paclitaxel, ifosfamide, irinotecan and imatinib and vinorelbine have been correlated to ERMBT results (Table 3.1).

The important role of CYP3A in biotransformation of docetaxel was reflected by a statistically significant correlation between ERMBT results and docetaxel clearance in three separate studies [35-37] (Table 3.1). However, in 19 metastatic non-small lung cancer patients receiving docetaxel, there were no correlations between the ERMBT and docetaxel pharmacokinetics [38].

In patients with severe liver disease before transplantation, $^{14}CO_2$ production from ^{14}C-erythromycin was just 16% of the mean value observed in 86 control patients without liver disease and the difference was highly significant (p=0.0001). After transplantation, ERMBT results improved dramatically, and the mean value was not significantly different from that observed in the controls [18]. Moreover, in patients undergoing ERMBT during liver transplantation, little $^{14}CO_2$ was produced during the anhepatic phase of the transplantation [39]. In accordance with these findings, ERMBT poorly correlated with docetaxel clearance in patients with normal liver function (R^2=0.03547; p=0.177) whereas significant correlation with unbound

Table 3.1 Clinical validity of the erythromycin breath test (ERMBT): Correlation with drug disposition of anticancer agents

ERMBT parameter	Drug parameter	N	R^2	p	Ref.
C_{20} (%dose/min)	Docetaxel clearance (L/h)	92	0.072	0.036	[63]
C_{20} (%dose/min)	Docetaxel clearance (L/h)	19	0.003	0.829	[38]
%^{14}C exhaled/h	Docetaxel clearance (L/h)	21	0.67	<0.0001	[35]
C_{20} (%dose/min)	Log transformed unbound docetaxel clearance (L/h) in patients with normal liver function	55	0.03547	0.177	[40]
C_{20} (%dose/min)	Log transformed unbound docetaxel clearance (L/h) in patients with abnormal liver function	22	0.603	<0.0001	[40]
C_{20} (%dose/min)	Docetaxel clearance (L/h)	54	0.19	0.0005	[37]
$1/t_{max}$	Docetaxel clearance (L/h)	54	0.15	0.003	[37]
C_{20} (% dose/min)	Docetaxel clearance (L/h)	20	ND	0.09	[36]
C_{20} (% dose/min)	Irinotecan clearance	30	0.078	0.136	[45]
ERMBT	Vinorelbine clearance	13	0.020	0.64	[43]
Log (AUC (0,1 h))	Paclitaxel AUC_{0-6} h: 0–6h	20	NA	0.47	[42]
C_{20} (% dose/min)	Ifosfamide AUC_{0-24} h: 0–24h	13	0.0002	0.96	[44]

Abbreviations: AUC, Area under the Curve; C_{20}, flux of exhaled $^{14}CO_2$ at t = 20 minutes; ERMBT, erythromycin breath test; $1/T_{max}$, the inverse of the time to maximum $^{14}CO_2$ flux ($1/T_{max}$). N, number of patients; P, p-value; R^2, variance of prediction error.

docetaxel clearance was demonstrated in patients with liver function abnormalities (R^2=0.603, p<0.0001) [40].

Paclitaxel is primarily metabolized by CYP2C8 into inactive metabolites, with a minor contribution of CYP3A4 [41]. In contrast to the CYP2C8 phenotype probe rosiglitazone, ERMBT was not statistically significant correlated to paclitaxel exposure AUC_{0-6h} in 20 patients receiving weekly paclitaxel [42].

In a phase I study with 25 patients with metastatic breast cancer receiving capecitabine and vinorelbine, there was no correlation between vinorelbine clearance (R^2=0.020, p=0.64), toxicity and ERMBT results [43]. In 13 sarcoma patients treated with doxorubicin and ifosfamide, ERMBT did not correlate with pharmacokinetics of ifosfamide and metabolites, nor with toxicity. The small sample size and the presence of the CYP3A inducer dexamethasone might have obscured the results [44].

CYP3A4 phenotype, as assessed by midazolam clearance, was significantly associated with irinotecan pharmacokinetics whereas ERMBT parameters were not, which might be due to a potential role of ABCB1 in the metabolism of irinotecan [45].

Clinical utility of ERMBT

The clinical utility of the ERMBT to predict toxicity from docetaxel has not been clarified as study results are conflicting. In 21 patients with metastatic sarcoma, the greatest toxicity related to docetaxel was observed in patients with the lowest ERMBT results [35]. In a study of 20 elderly patients treated with weekly docetaxel there is a significant correlation between ERMBT results and docetaxel clearance but not with frequency of ≥grade 3 toxicity was reported. Extrapolation of results from this study is limited by the small sample size and accrual of patients from a single center [36]. In a study with 19 metastatic non-small cell lung cancer patients, ERMBT parameters were not correlated to ANC nadir counts in patients receiving 3-weekly docetaxel [38]. In conclusion, studies on correlations between ERMBT and docetaxel clearance are inconsistent. So far, ERMBT has no clinical utility, as clinical rules including ERMBT results predicting docetaxel dose and/or toxicity are lacking.
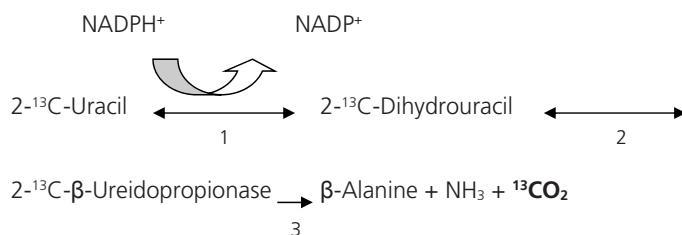
3. 2-^{13}C-URACIL BREATH TEST

Introduction

Fluoropyrimidines are frequently prescribed anticancer drugs for colorectal and breast cancer and include 5-fluorouracil (5-FU) and the prodrug capecitabine [46]. Most patients tolerate fluoropyrimidines well, but about 10% develop severe, potentially life-threatening complications. The most important cause of these complications is a deficiency of dihydropyrimidine dehydrogenase (DPD), the primary enzyme that detoxifies fluoropyrimidines, converting >80% of an administered dose of 5-FU to inactive metabolites [47]. It is estimated that DPD deficiency is four times more prevalent in African American population (8%), in particular women, compared with Caucasians (2.8%, p=0.07) [48].

A strong positive association has been demonstrated between single nucleotide polymorphisms (IVS14+1G>A and 2846A>T) in *DYPD*, the gene encoding for DPD, and toxicity of capecitabine in patients with metastatic colorectal cancer [49]. So far, the gold standard for detection of DPD deficiency in patients is the measurement of DPD activity in peripheral blood mononuclear cells (PBMCs) by radioenzymatic assay [50]. Although there is a good correlation between 5-FU clearance and DPD activity in PBMCs [50], the method is time consuming and expensive.

Alternatively, a uracil breath test (UraBT) has been developed , using 2-^{13}C-uracil for a DPD phenotype probe. The principle of the UraBT is based on the production of $^{13}CO_2$ from 2-^{13}C-uracil by

NADPH$^+$ NADP$^+$

2-^{13}C-Uracil   1   2-^{13}C-Dihydrouracil   2

2-^{13}C-β-Ureidopropionase → β-Alanine + NH$_3$ + ^{13}CO$_2$
       3

1: Dihydropyrimidine dehydrogenase

2: Dihydropyrimidinase

3: β-Ureidopropionase

Figure 3.2   **Principle of Uracil breath test: eventually, ^{13}CO$_2$ is formed and detected by IR spectrophotometry.** Abbreviations: NADP, Nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of Nicotinamide adenine dinucleotide phosphate.

the enzymes of the pyrimidine-catabolic pathway (Figure 3.2) [51]. ^{13}CO$_2$ and ^{12}CO$_2$ concentrations in breath samples are measured by infrared spectrometry. The amount of ^{13}CO$_2$ present in breath samples is expressed as a delta-over baseline (DOB) ratio that represents a change in the ^{13}CO$_2$/^{12}CO$_2$ ratio of breath samples collected before and after 2-^{13}C-uracil ingestion [51].

In DPD-deficient individuals, decreased exhaled ^{13}CO$_2$ levels represented reduced 2-^{13}C-uracil metabolism [51]. In this study, a single time-point measurement (DOB$_{50}$) was used to discriminate between normal DPD and DPD deficient subjects.

Correlation of UraBT with genotype and other DPD phenotype tests

In a study of 50 subjects with a normal DPD status, 7 with a partially, and 1 with a profound DPD-deficiency assessed by DPD radio assay and *DYPD* genotype, all eight DPD-deficient individuals were correctly identified (100% sensitivity). In addition, 48 of 50 individuals with normal DPD activity were also correctly identified (96% specificity). In a study with 255 African-American and Caucasian healthy volunteers, the UraBT demonstrated 99.2% specificity and 85.7% sensitivity for detecting DPD deficiency [48]. In a study with 23 healthy volunteers and 8 cancer patients, UraBT DOB$_{50}$ significantly correlated with PBMC DPD activity (R^2=0.608), plasma [2-(^{13}C]uracil area under the curve (R^2=0.533) and 2-^{13}C-dihydrouracil appearance rate (R^2=0.578), suggesting that UraBT might predict DPD deficiency [52].

Clinical utility of UraBT

One study demonstrated the value of UraBt in a clinical setting with the drug S-1, combining the antitumor activity of oral tegafur (pro-drug of 5-fluorouracil (5-FU) with DPD inhibition of 5-chloro-2,4-dihydroxypyridine (CDHP). UraBT correlated significantly (R^2=0.303, p=0.002) with 5-FU plasma concentrations and in a multivariate analysis, only UraBT was an independent predictor of anticancer response to S1 [53].

In conclusion, UraBT has thus far been demonstrated to be a reliable test for the rapid detection of DPD deficiency in healthy volunteers. However, the UraBT to demonstrate its value in predicting DPD status in cancer patients, has not transitioned its way from the research stage to the clinic yet.

4. ^{13}C-DEXTROMETHORPHAN BREATH TEST

Introduction

In oncology, CYP2D6 is involved in metabolism of gefitinib [54] and tamoxifen [55;56]. Tamoxifen is activated by CYP2D6 to 4-hydroxytamoxifen, which has a 50–100-fold higher antiestrogen activity compared to tamoxifen [55;56]. More importantly, CYP2D6 is the main enzyme for the activation of *N*-desmethyltamoxifen to endoxifen, the most active metabolite of tamoxifen. Variations in endoxifen concentrations have been attributed to expression of variant *CYP2D6* alleles and might explain the variation in response to tamoxifen. Although several studies demonstrated that patients with CYP2D6 PM phenotype had 2 to 4-fold lower endoxifen levels compared to extensive metabolizers (EM), only one study has been published [56] which suggests a correlation between endoxifen levels and breast cancer recurrence rate. Therefore, endoxifen levels may serve as a predictor for clinical outcome.

Leeder et al. [13] developed a single-point ^{13}C-dextromethorphan breath test (DM-BT) to phenotype CYP2D6 in a simple, rapid way.

^{13}C-labeled DM is dependent on CYP2D6 *O*-demethylation. The released methyl group is involved in the formation of $^{13}CO_2$ that is released in expired breath over time (Figure 3.1).

Correlation with *CYP2D6* genotype and other CYP2D6 phenotype tests

DM-BT results in 30 healthy subjects correlated well with urinary ^{13}C-dextromethorphan/dextrorphan (DM/DX) ratio, an alternative way to phenotype CYP2D6 . By defining CYP2D6 poor metabolizer (PM) phenotype (DOB_{50} ≤0.5), sensitivity and specificity of DM-BT to detect PM was 100%, and 95% respectively using either genotype or DM/DX ratio as the "gold" standard [13].

Clinical utility of DM-BT

In a recent prospective study, Graan et al. found a strong negative correlation (R^2=0.518, p<0.001) between dextromethorphan exposure after administering a single oral dose of dextromethorphan and endoxifen levels in women with invasive breast cancer who used 40 mg of tamoxifen. In 65 patients with early breast cancer using tamoxifen, CYP2D6 phenotype was assessed by DM-BT [57]. CYP2D6 phenotype determined by the DM-BT explained variation in serum steady-state endoxifen levels for 47.5% (R^2=0.475, p<0.001).

Positive and negative predictive values for a recently suggested threshold serum level of endoxifen (5.97 ng/ml) for increased breast cancer recurrence rate [56] were 100% and 90% respectively for DM-BT DOB_{50} values of 0.7–0.9, emphasizing the possible role of DM-BT in clinical practice for personalizing endocrine therapy.

However, correlation between DM-BT and clinical outcome (breast cancer recurrence rate) needs to be evaluated in future prospective [64] studies before DM-BT could be used for phenotyping CYP2D6 in breast cancer patients prior to initiating endocrine therapy with tamoxifen or aromatase inhibitors.

5. ^{13}C-PANTOPRAZOLE BREATH TEST

Recently, pantoprazole-^{13}C breath test (Ptz-BT) has been introduced to phenotype CYP2C19 mediated drug metabolism [58]. Cyclophosphamide (CP), a widely used cytostatic drug, is metabolized by polymorphic drug metabolizing enzyme CYP2C19. Analysis of variance revealed that the CYP2C19*2 genotype influenced significantly pharmacokinetics of CP at doses ≤1000 mg/m^2 [59]. In another study the loss of function allele for CYP2C19 appeared to result in decreased bioactivation of cyclophosphamide both *in vitro* and in patients [60]. Studies have to confirm whether Ptz-BT might be used for selection of the optimal dose of cyclophosphamide.

6. DISCUSSION

Determination of the phenotype by BT, might enable clinicians to personalize medicine in order to improve outcome in cancer patients. Pharmacogenomics was introduced in clinical practice over the last 10 years and many labels of approved drugs contain information regarding genetic testing to improve treatment and minimize risks. For 6-mercaptopurine, irinotecan, carbamazepine, abacavir, warfarin, clopidogrel, cisplatin, and pimozide, pharmacogenetic tests have been described in the drug label [61]. Thus, when there is a clear relationship between genotype variants and toxicity or response, genotype-guided dosing may be considered. However, for many drugs, like antihypertensives, proton-pump-inhibitors and psychiatric medications, a genetic biomarker is not available to guide for efficacy and/or safety.

Therapeutic drug monitoring (TDM) of a specific drug is a relatively simple way to determine the phenotype in patients using medications for which a validated analytical method is available and results may lead to dose adjustments. However, the use of TDM has been limited in the field of oncology, for the reason that therapeutic indices for most anticancer agents have not been established thus far [62]. Besides, TDM is laborious and expensive and might not have been validated in clinical practice. Moreover, the phenotype might change with advancing age and/or disease necessitating repetition of individual analysis over time. Recently, over the past ten years, and as shown in this review, single point breath tests have been researched, enabling a physician to obtain BT results in 50 minutes. In oncology, BT are of particular interest because most treatments display high toxicity and patients may be frail. Therefore, in theory, BT may reduce toxicity and improve efficacy through individualizing treatment by means of introducing a phenotype guided treatment and/or dosing. To minimize health risks, radioactive ^{14}C labeled probes have been replaced by low-costs ^{13}C-labeled substrates.

UraBT might become the most promising BT to be introduced in clinical oncology as DPD deficiency is a known biomarker for fluoropyrimidine associated toxicity. However, UraBT has not found its way to the clinic yet, as an upfront DPD phenotype test for fluoropyrimidine treated cancer patients, probably because data on clinical and pharmacoeconomic performance are lacking thus far.

ERMBT was the first breath test developed by Watkins to predict CYP3A phenotype in subjects, using ^{14}C labeled erythromycin. Despite the improvements of the ERMBT clinical value has not been shown: studies on correlation of ERMBT results with pharmacokinetics of anticancer agents like docetaxel, ifosfamide and vinorelbline are inconsistent and clinical experience with the test

is very limited. Most importantly, clinical utility of ERMBT has not been demonstrated in 2 of the 3 studies correlating ERMBT results to docetaxel toxicity [36;38].

Results of DM-BT however, might be more optimistic as DM-BT studies show good results regarding probe selectivity and clinical utility. Despite a good correlation of DM-BT with endoxifen levels in breast cancer patients, studies on DM-BT guided tamoxifen related breast cancer outcome, need to be carried out. Thus far, DM-BT plays no role in clinical decision making whether to treat breast cancer patients with CYP2D6 PM phenotype with alternative aromatase inhibitors instead of tamoxifen.

In conclusion, despite hopeful results of certain BTs to identify patients with metabolic or genetic risks for undertreatment and or treatment related toxicity, there is no evidence for the usefulness of BTs in a clinical oncology setting at this time. The demands imposed to upfront phenotype tests in drug therapy are high. Along with the validation of test performance, clinical utility must be proven in terms of reliable prediction of (positive or negative) clinical outcome such as PFS, OS or toxicity.

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