GENE EXPRESSION IN BUCCAL KERATINOCYTES WITH EMPHASIS ON CARBONYL METABOLISM

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Stockholm 2008
Une sortie, c'est une entrée que l'on prend dans l'autre sens.

Boris Vian
ABSTRACT

The inner lining of the cheek, the buccal mucosa, is a target for air-borne, dietary and tobacco usage-derived carcinogens, but also interesting from a drug delivery point of view. Cancer arising in the buccal epithelium, buccal squamous cell carcinoma (BSCC), often diagnosed at a late disease stage, is highly aggressive and recurrent, emphasizing the need for novel approaches in diagnosis and therapy.

An in vitro model for human buccal carcinogenesis consisting of normal buccal keratinocytes (NBK) and two transformed cell lines of buccal origin was applied to explore mechanisms of buccal carcinogenesis, tumor marker and drug target expression. Two-dimensional gel electrophoresis, DNA microarray analysis, and the application of three bioinformatics programs for data mining allowed for the identification of multiple established and potential novel markers for BSCC, including tumor promoter/suppressor genes. Furthermore, post-confluent culture of NBK in absence and presence of fetal bovine serum was successfully used to induce terminal squamous differentiation (TSD) to various extents and thus enrich for different strata of the epithelium. Here, expression and activity of carbonyl-metabolizing enzymes (CMEs) were assessed in view of their multiple roles in phase I biotransformation. The combined results put forward ADH3 transcription as a proliferation marker as well as ALDH4A1, AKR1A1 and HPGD transcription as markers for the onset of TSD. Finally, the findings suggested increased CME-mediated xenobiotic activity at the onset of TSD, with potential implications for drug response and BSCC susceptibility.

In addition to its function as a ubiquitous formaldehyde scavenger, including in the buccal mucosa, alcohol dehydrogenase 3 (ADH3) irreversibly reduces S-nitroso-glutathione (GSNO) which implies an important role in nitric oxide homeostasis. Assessment of ADH3 in terms of formaldehyde/GSNO competition provided indirect evidence for formaldehyde as a physiological trigger of ADH3-mediated GSNO depletion with possible direct implications for asthma, where formaldehyde is known for exacerbating and GSNO for protective effects. Moreover, GSNO reduction resulted in the glutathione-controlled formation of glutathione transferase inhibitors, thus possibly affecting phase II biotransformation under conditions of oxidative stress. In light of these ADH3-mediated adverse effects, several substrate analogues were tested as ADH3 inhibitors. The results provide guidelines for future design of a specific ADH3 inhibitor with potential direct clinical use for the prevention of asthma-exacerbating effects.
LIST OF PUBLICATIONS

This thesis is based on the following articles, referred to in the text by their Roman numerals.


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LIST OF ABBREVIATIONS

ACAT1  Acetyl-CoA acetyltransferase 1
ADH    Alcohol dehydrogenase
AKR    Aldo-keto reductase
ALDH   Aldehyde dehydrogenase
BSCC   Buccal squamous cell carcinoma
CBR    Carbonyl reductase
CDC2   Cell division control protein 2
CFE    Colony-forming efficiency
CK     Cytokeratin
CME    Carbonyl-metabolizing enzyme
CYP    Cytochrome P450
2D-PAGE Two-dimensional polyacrylamide gel electrophoresis
EGF    Epidermal growth factor
ESI MS Electrospray ionization mass spectrometry
ESTD   Esterase D
FBS    Fetal bovine serum
GO     Gene Ontology
GOTM   Gene Ontology Tree Machine
GSH    Glutathione
GSNO   S-nitrosoglutathione
GSSG   Glutathione disulfide
GST    Glutathione S-transferase
HMGSH  S-Hydroxymethylglutathione
hnRNP  Heterogeneous nuclear ribonucleoprotein
HPGD   15-(NAD\(^+\))-hydroxyprostaglandin dehydrogenase
HPV    Human papillomavirus
HSD11B1 11-\(\beta\)-hydroxysteroid dehydrogenase type 1
HSP    Heat shock protein
IPA    Ingenuity pathway analysis
IVL    Involucrin
MALDI-TOF MS Matrix-assisted laser desorption/ionization - time-of-flight mass spectrometry
MDR    Medium-chain dehydrogenases/reductases
NBK    Normal buccal keratinocytes
NNK    4-(M ethylnitrosamino)-1-(3-pyridyl)-1-butanone
NO     Nitric oxide
NQO    NAD(P)H:quinone acceptor oxidoreductase
PAH    Polycyclic aromatic hydrocarbon
PCNA   Proliferating cell nuclear antigen
QOR    Quinone oxidoreductase
SCC    Squamous cell carcinoma
SDR    Short-chain dehydrogenases/reductases
SFN    Stratifin
SNO    S-nitrosothiol
### Abbreviations

- **SPR**: Small proline-rich protein
- **Start-PCR**: Standardized reverse transcriptase-polymerase chain reaction
- **SV40**: Simian Virus 40
- **SV40T**: Simian Virus 40 large T antigen
- **TGF**: Transforming growth factor
- **TGM**: Transglutaminase
- **TSD**: Terminal squamous differentiation

### Amino Acid Codes

<table>
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<th>Amino Acid</th>
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1 INTRODUCTION

The inner lining of the cheek, the buccal mucosa, is continuously exposed to inhalatory and dietary carcinogens. Cancer arising in the buccal epithelium, buccal squamous cell carcinoma (BSCC), belongs to the most aggressive and recurrent oral cancer types [1, 2]. Despite advances in clinical assessment and treatment methods, survival rates have not improved, mostly due to diagnosis at late disease stages and cancer therapy resistance [3-5]. Hence, there is a clear need for novel approaches in diagnosis and therapy.

In order to develop and assess new strategies, robust and reproducible test model systems for buccal carcinogenesis are necessary. To this end, the present thesis involved the use of an in vitro model of buccal carcinogenesis consisting of normal buccal keratinocytes (NBK) and two transformed buccal keratinocyte lines termed SVpgC2a and SqCC/Y1, which show characteristics of premalignancy and full-blown malignancy, respectively [6-10]. Furthermore, standardized serum-free culture conditions established for NBK allow for the use of fetal bovine serum (FBS) as inducer of terminal squamous differentiation (TSD), the major pathway of programmed cell death in the buccal epithelium [11, 12]. This permits the culture of differentiated, suprabasal-like NBK in addition to highly proliferative, basal-like NBK. Notably, SVpgC2a and SqCC/Y1 can be cultured under identical conditions, but have acquired TSD-deficiency in the process of immortalization/malignant transformation [7, 12].

In this context, the characterization of xenobiotic metabolism is of particular interest for several reasons. Obviously, elucidation of detoxification and carcinogen activation mechanisms in the normal buccal mucosa can help us understand susceptibility to BSCC. Additionally, knowledge about xenobiotic metabolism in the buccal mucosa is important for its use as drug delivery site [13, 14]. Moreover, xenobiotic enzymes that exhibit altered expression in a (pre-)malignant tissue state might qualify as drug targets, with the use of specifically designed drugs that can either be neutralized in normal cells or activated in (pre-)malignant cells. Finally, transformed cell lines, when used as models for the respective normal tissue in toxicological studies, generally need to be evaluated in terms of metabolic competence which can differ considerably from the cell type of origin [15].
Carbonyl metabolizing enzymes (CMEs), i.e. enzymes that are involved in the metabolism of carbonyl-group containing compounds, play an important role in human xenobiotic metabolism [16]. CMEs convert exogenous substrates that are highly relevant for normal as well as neoplastic buccal epithelium, as they contribute to the metabolism of oral cancer risk factors, e.g. ethanol, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and polycyclic aromatic hydrocarbons (PAH), as well as anti-cancer therapeutics, e.g. retinoids and anthracyclines [17-23]. The simplest carbonyl compound, formaldehyde, has recently been classified as a carcinogen [24]. Among all CMEs, alcohol dehydrogenase 3 (ADH3) is the most efficient and widespread formaldehyde scavenger in human tissues including the buccal mucosa and the described in vitro model systems thereof [25-27]. During the last decade, evidence has accumulated that suggests an additional important role for ADH3 in endogenous nitric oxide (NO) homeostasis [28-32]. In fact, many CMEs are equally involved in exogenous and endogenous metabolism, but few have been investigated in terms of exogenous/endogenous substrate competition, a subject of great toxicological and pharmacological interest [33].

Within this thesis work, gene expression was assessed in the described in vitro model for buccal carcinogenesis and evaluated using bioinformatics tools with the long-term aim to elucidate mechanisms of carcinogenesis, find novel tumor markers and, finally, propose novel diagnostic strategies. The studies included a global expression assessment (Paper I) as well as two targeted approaches for the investigation of CME expression, including ADH3 transcription, in proliferative and differentiated buccal keratinocytes (Papers II and III). Finally, two studies deal with the enzyme ADH3 and implications of its dual function as formaldehyde dehydrogenase and S-nitrosoglutathione (GSNO) reductase (Papers IV and V).
2 BACKGROUND

2.1 THE BUCCAL MUCOSA

As a major portal of entry, the oral cavity is an evident target for air-borne, dietary and tobacco usage-derived carcinogens. The inner linings of the oral cavity can be divided into three major categories: 1) Keratinizing mucosae, including the masticatory mucosae of the gingiva and the hard palate; 2) non-keratinizing mucosae, including the buccal mucosa, the floor of the mouth, and the mucosa of the esophagus; 3) the dorsum of the tongue, which is covered by a specialized mucosa [34]. Non-keratinizing mucosae as the buccal lining exhibit greater permeability than keratinizing mucosae, which is attributed to differences in intercellular lipid rather than degree of keratinization [13]. This property makes the buccal epithelium and the underlying tissue particularly vulnerable for carcinogenic compounds, but has also lead to a growing interest in the buccal mucosa as a drug delivery site [14].

2.1.1 Structure of the buccal mucosa

Fig. 1. Structure of the buccal mucosa, modified from Harris and Robinson [35].
The buccal mucosa is constituted of a stratified squamous epithelium supported by a fibrous connective tissue, more specifically called the lamina propria mucosae (Fig. 1). These two tissue compartments are separated by the basement membrane, an extracellular matrix constituted of a network of collagens, laminins and other proteins [36]. The present thesis deals with cultured cells of the buccal epithelium. As all stratified squamous epithelia, this epithelium serves to protect the underlying tissue from mechanical, physical, chemical and microbial insult and therefore must undergo constant repair and renewal [37]. The different keratinocyte layers can be subdivided into three parts, the basal layer, the suprabasal layers and the superficial layers (Fig. 1) [14, 34, 38]. The innermost basal layer harbors amplifying cells and a small proportion of infrequently dividing stem cells, which together form the basis of the proliferative potential of the tissue. Normal tissue homeostasis is dependent on a fine-tuned balance between proliferation and a well-defined terminal differentiation program called terminal squamous differentiation (TSD) [34].

2.1.2 Terminal squamous differentiation (TSD)

TSD is the major pathway of cell death in all stratified epithelia. As keratinocytes mature, they migrate from basal to suprabasal and superficial layers, while they gradually lose their proliferative ability and undergo TSD until finally dead cells are shed off from the outmost layer [34]. In keratinizing epithelia, TSD leads to the production of the outmost stratum corneum, a layer of flat cornified cells densely filled with cytokeratin (CK) filaments and surrounded by a thickened cell envelope. The process of TSD in non-keratinizing epithelia including the buccal subsite is also accompanied by morphological changes, albeit of less distinct character (Fig. 2) [34]. As cells differentiate, they gradually enlarge, flatten and form a cross-linked protein envelope, a modified or “incomplete” version of the tougher cornified envelope found in keratinizing epithelia. Precursors or inactive forms of protein envelope components are already expressed in suprabasal cells while the assembly mainly takes place in the surface cells. Also the composition of CKs, the predominant cytoskeletal proteins in all kinds of epithelia, changes during the process of TSD. At the same time, expression of proteins that associate with proliferation is gradually downregulated [34, 37, 38]. The median turnover time of the buccal epithelium is 14 days [34]. Various proteins differentially expressed during buccal TSD and their functions are given in Table 1; their approximate distribution in the buccal epithelium is illustrated in Fig. 2.
**Table 1:** Proteins differentially expressed during TSD [37-41].

<table>
<thead>
<tr>
<th>Protein (Abbr.)</th>
<th>Gene</th>
<th>Cellular component</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin 5 (CK5)</td>
<td>KRT5</td>
<td>Cytoskeleton</td>
<td>Components of intermediate filaments</td>
</tr>
<tr>
<td>Cytokeratin 14 (CK14)</td>
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<tr>
<td>Involucrin (IVL)</td>
<td>IVL</td>
<td></td>
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<tr>
<td>Small proline-rich protein 1 (SPR1)</td>
<td>SPRR1</td>
<td>Crosslinked protein envelope</td>
<td>Components of the crosslinked protein envelope</td>
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<tr>
<td>Small proline-rich protein 3 (SPR3)</td>
<td>SPRR3</td>
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<tr>
<td>Transglutaminase 1 (TGM1)</td>
<td>TGM1</td>
<td></td>
<td>Crosslinking of components of the protein envelope</td>
</tr>
</tbody>
</table>

**Fig. 2.** Structure of the buccal epithelium. Ongoing processes and proteins associated with different layers are indicated. Protein abbreviations are as in Table 1. PCNA, Proliferating cell nuclear antigen; CDC2, Cell division control protein 2

Regulation of tissue homeostasis in stratified epithelia relies on the action of various cytokines including such that stimulate proliferation, e.g. epidermal growth factor.
(EGF) and transforming growth factor α (TGF-α), as well as such that induce TSD, e.g. TGF-β and interferon-γ [11, 34, 42]. Furthermore, a range of low-molecular compounds induce or modulate keratinocyte differentiation, e.g. retinoids, triiodothyronine, vitamin D₃ and glucocorticoids [43-46]. Finally, the presence of a calcium gradient in stratified epithelia in vivo suggests the involvement of calcium in the induction or regulation of TSD [47]. Indeed, calcium is often used as inducer of epidermal differentiation in vitro and can equally induce certain, notably not all, characteristics of TSD in cultured buccal keratinocytes [9, 42].

2.1.3 Buccal squamous cell carcinoma (BSCC)

Oral cavity cancer is the 8th most common cancer type world-wide and thus a major global health problem [48]. More than 95% of all mouth neoplasms are squamous cell carcinomas (SCCs), meaning that they arise in epithelia [49-51]. BSCC is characterized by a high tendency of locoregional recurrence and poor survival rates and thus considered one of the most aggressive oral cancer types [1, 2, 52]. Although relatively rare in most Western countries, BSCC belongs to the most common cancer forms in South and Southeast Asia [48, 51, 53]. This is in large due to the prevalence of betel quid chewing, which undoubtedly represents one of the major risk factors for BSCC [53-56]. Other important risk factors include tobacco chewing and smoking, alcohol abuse, dietary deficiencies and viruses, particularly human papillomavirus (HPV) [55-58].

Currently, diagnosis of oral (pre-)malignant lesions still mostly relies on visual inspection including clinical staging and microscopic assessment of tissue specimens and cells [3, 56, 59, 60]. The prognostic value of molecular markers is subject of extensive current research, but so far neither an individual marker nor a panel of markers has been determined that is more reliable than the above mentioned conventional methods [3, 61, 62]. However, the establishment of laser-capture microdissection, array technologies and other -omics techniques for diagnostic tumor profiling is being pursued and there is no doubt that molecular markers have a strong potential in future oral cancer prognosis as well as individualized therapy [63-65].

Retinoids, β-carotene, α-tocopherol and ascorbic acid have been used in chemopreventive studies of oral premalignant lesions, i.e. in efforts to suppress malignant transformation and reverse lesions to a normal state, albeit with inconsistent
outcome [4, 66, 67]. Oral SCCs are mainly treated by surgical resection, radiotherapy, chemotherapy, or a combination thereof. Novel treatment strategies are being developed and evaluated, including targeted molecular therapies, e.g. antibodies against overexpressed tumor promoters and gene transfer of tumor suppressor genes [66, 68-70].

2.1.4 Oral carcinogenesis

Few studies with an emphasis on human buccal carcinogenesis have been performed. Carcinogenesis of the buccal subsite has largely been studied in the carcinogen-induced hamster cheek pouch carcinogenesis model [71]. However, the hamster cheek pouch epithelium is considerably different from its human counterpart, both regarding morphology and carcinogen susceptibility, which complicates interspecies extrapolation and emphasizes the need for suitable human models [72].

Generally, oral carcinogenesis is a combined result of an individual’s genetic predisposition and exposure to carcinogens and considered to be a multistep process (Fig. 3). First, a cell undergoes initiation by an initial genetic insult affecting a central tumor promoting or suppressor gene, e.g. a mutation, chromosomal insertion/deletion or change in DNA ploidy. Frequently overexpressed proteins in oral SCCs include such that mediate self-sufficiency in growth signals, e.g. EGF receptor and transcription factors of the Myc family [61-63, 70]. On the contrary, inactivation of tumor suppressor genes leads to acquisition of insensitivity to anti-growth signals and evasion of apoptosis. In oral SCCs, RB1 (encoding retinoblastoma-associated protein, Rb), TP53 (encoding p53) and CDKN2A (encoding p16) are among the loci that are most often altered, mutated or lost [61, 63, 70]. Initiation is followed by promotion involving the formation of a cluster of preneoplastic cells, a so-called premalignant lesion, which is in principle still reversible. The acquisition of further genetic alterations and increasing genetic instability leads to conversion to a malignant phenotype and finally progression into metastatic cancer [70, 72].

Also viral infection can be the cause of genetic alterations in such key regulator genes. Multiple lines of evidence suggest that mucosal HPV is involved in the etiology of oral SCC [73]. Its oncogenic properties are mainly attributed to the actions of two viral proteins, E6 and E7, which among others inhibit activities of p53 and Rb, respectively [58, 74]. Studies on the involvement of other viruses, e.g. Epstein-Barr
virus and hepatitis C virus have provided inconclusive results [75]. Notably, simian virus 40 (SV40), originally discovered about 50 years ago as a contaminant in polio vaccine stocks, expresses SV40 large T antigen (SV40T), a protein that binds and inactivates p53 and Rb, similarly to the E6/E7 proteins of HPV [76]. This property is sufficient to immortalize epithelial cells in vitro [77]. However, a causal role of SV40 in human cancers is still under debate [76].

**Fig. 3.** Oral carcinogenesis is a multistep process [72].

### 2.1.5 In vitro models for the oral mucosa

In vitro models for normal and transformed states of the oral mucosa are widely used for studies of toxicology, carcinogenesis mechanisms and chemoprevention. Notably, there are only very few cell culture models for the buccal mucosa [72, 78]. In principle, the use of normal oral keratinocytes would be the optimal choice for any kind of in vitro study on normal keratinocyte biology including toxicity testing. But all normal oral keratinocytes exhibit a finite life span due to the fact that they have retained the ability to undergo TSD in culture, which results in a natural limit in cell numbers. Hence, investigators often resort to transformed cell lines instead, including experimentally immortalized cell lines or such derived from (pre)malignant lesions, as they can be relatively effortlessly, reproducibly and limitlessly cultured [79]. However, besides the gained ability to circumvent normal cell death, the immortalization process also often results in metabolic deficiencies or alterations which make the extrapolation to normal cells difficult [15]. Thus, if used for toxicology testing, continuous cell lines should optimally be evaluated in terms of relevant metabolic competence.
Immortal oral keratinocyte lines have been established from dysplastic human oral mucosa as well as by infection with oncogenic viral antigens from HPV or SV40. As such cell lines often exhibit a premalignant-like, yet non-tumorigenic, phenotype, they can be good model systems to study chemoprevention or malignant transformation [8, 72, 78, 80]. Notably, the SV40T-immortalized cell line used within this thesis work, SVpgC2a (described in more detail below), is the only existent immortalized human cell line from the buccal subsite. Numerous tumor cell lines have been derived from various oral SCCs including a few from BSCC. These cell lines are commonly tumorigenic when injected into immunodeficient mice and thus can be considered models for full-blown malignancy [72, 78, 79]. The buccal tumor cell line SqCC/Y1, used within this thesis work, will be discussed in more detail below [6, 9].

Hence, a combination of normal oral keratinocytes, premalignant-like and tumor-derived cell lines has the potential to qualify for studies of oral cancer progression (cf. Fig. 3). In this context, however, a frequent impediment is the fact that immortalized/premalignant and malignant oral tumor cell lines are usually established and cultured under addition of FBS to the culture medium [72, 78, 79]. In contrast, normal oral keratinocytes are nowadays commonly cultured under serum-free conditions. These discrepancies in culture medium composition complicate in vitro studies on carcinogenesis substantially.

2.1.6 NBK, SVpgC2a and SqCC/Y1

Monolayer cultures of NBK can be grown from buccal biopsies obtained by maxillofacial surgery. Standardized serum-free conditions have several advantages: First, they omit the problems associated with FBS batch variations which improves reproducibility [12, 72]. Furthermore, FBS is a very efficient inducer of TSD, which probably reflects the involvement of multiple agents, as discussed above [9, 11, 42, 46, 72, 81]. Hence, omitting serum from the culture conditions helps NBK maintain a highly proliferative phenotype, which improves final cell yield [11, 72]. Importantly, the same standardized serum-free culture conditions used for NBK are applicable to SVpgC2a and SqCC/Y1. The morphologies of the three cell types cultured under serum-free conditions are presented in Fig. 4 and their properties are summarized in Table 2.
The cell line SVpgC2a was obtained by transformation of NBK (non-smoking 17-year-old donor) with a plasmid carrying the SV40T gene. The SV40T gene was integrated in the genome, stably expressed and the protein formed complexes with the wild-type p53 protein [77]. The resulting cell line was apparently immortal, exhibited a highly proliferative phenotype and yet was non-tumorigenic when injected into immunodeficient mice [77, 82]. SVpgC2a is TSD-deficient, i.e. shows decreased responsiveness to TSD-inducing agents including decreased growth inhibition and expression of markers of TSD. Furthermore, SVpgC2a displays a hypodiploid karyotype which is relatively stable throughout prolonged culture [83]. Finally, the keratin profile of SVpgC2a reminds of severe epithelial dysplasia, a typical feature of a premalignant lesion [10].

The cell line SqCC/Y1 is originally derived from a verrucous squamous cell carcinoma of a female patient and was established under conditions using serum, but later adapted to the serum-free culture conditions used for NBK [9]. The single p53 allele carries two missense mutations and is not expressed [6]. SqCC/Y1 is immortal, produces tumors when injected into immunodeficient mice, is resistant to induction of growth-arrest/TSD and shows decreased expression of TSD markers in response to TSD-inducing agents. Furthermore, with 63-83 chromosomes/metaphase SqCC/Y1 displays an aneuploid karyotype [9].

Fig. 4. Morphology of monolayer cultures of human buccal keratinocytes. (A) NBK show typical cobblestone-like epithelial morphology. Some terminally differentiated cells with enlarged cell surface are present. (B) SVpgC2a show elongated fibroblast-like morphology. (C) SqCC/Y1 show cells of variable size and polygonal cell shape.
Table 2: Properties of the three cell types used within this thesis work [6-9, 12, 77].

<table>
<thead>
<tr>
<th>Property</th>
<th>NBK</th>
<th>SVpgC2a</th>
<th>SqCC/Y1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Establishment</td>
<td>derived from biopsies following maxillofacial reconstructural surgery</td>
<td>derived from SV40T-transfection of NBK</td>
<td>derived from a buccal squamous cell carcinoma</td>
</tr>
<tr>
<td>Longevity</td>
<td>cultured for ≈ 3-4 weeks until 80% confluency after 2nd passage</td>
<td>immortal, &gt;1000 population doublings</td>
<td>immortal, &gt;1000 population doublings</td>
</tr>
<tr>
<td>p53 status</td>
<td>wild-type</td>
<td>wild-type p53 expressed but inactivated by SV40T</td>
<td>one single p53 allele with two missense mutations</td>
</tr>
<tr>
<td>Tumorigenicity</td>
<td>non-tumorigenic</td>
<td>non-tumorigenic</td>
<td>tumorigenic</td>
</tr>
<tr>
<td>TSD</td>
<td>undergoes TSD in culture</td>
<td>TSD-deficient</td>
<td>TSD-deficient</td>
</tr>
<tr>
<td>Karyotype</td>
<td>normal</td>
<td>hypodiploid (39-45 chromosomes/metaphase)</td>
<td>aneuploid (63-83 chromosomes/metaphase)</td>
</tr>
</tbody>
</table>

2.2 XENOBIOTIC METABOLISM

Xenobiotic metabolism describes the metabolism of exogenous compounds that are foreign to an organism’s normal biochemistry, i.e. drugs and toxins that are not part of the normal diet. Absorption of most foreign compounds into the human body is by passive diffusion through the lipid bilayer of cell membranes; hence, most potentially toxic compounds are lipid soluble. Lipophilic compounds are readily reabsorbed after biliary excretion which complicates and delays their final elimination. In order to facilitate excretion, such compounds need to be converted to more hydrophilic compounds, a task which is accomplished by a set of metabolic pathways collectively referred to as biotransformation.

2.2.1 Reactions and enzymes in xenobiotic metabolism

Biotransformation is divided into three phases and involves a large variety of reactions and enzymes. Phase I reactions aim at introducing functional groups into
xenobiotic compounds which enables subsequent phase II reactions, i.e. conjugation reactions with hydrophilic compounds. Oxidations and reductions are among the most important types of phase I reactions. The majority of xenobiotic oxidations are executed by microsomal mono-oxygenases including the cytochrome P450 (CYP) system and the flavin-containing mono-oxygenases. Reductions can be catalyzed by both microsomal and cytosolic enzymes. For instance, cytotoxic quinones can be reduced by microsomal NADPH cytochrome P-450 reductase, but also by several cytosolic enzymes including carbonyl reductase 1 (CBR1) and NAD(P)H:quinone acceptor oxidoreductases (NQOs) [84-86]. The latter belongs to the group of xenobiotic carbonyl-metabolizing enzymes (CMEs) which will be discussed in more detail below [16]. A phase II reaction consists in the conjugation of the exogenous compound with an endogenous hydrophilic compound. For example, conjugation with glutathione (GSH) is catalyzed by glutathione transferases (GSTs), again involving both microsomal and cytosolic enzymes. Phase III reactions refer to the processing of a GSH conjugate as e.g. in the mercapturate pathway which yields the N-acetyl-cysteinyln conjugate (= mercapturate) as final product [87].

Notably, biotransformation does not necessarily lead to the detoxification, i.e. disposal of a foreign compound by excretion, but can actually underlie the toxicity of a compound by producing a chemically reactive metabolite. This process known as metabolic activation or bioactivation can be taken advantage of for cancer therapy with the design of tumor-activated prodrugs [88]. There often exist competitive metabolic pathways for a compound, some of which may be detoxification pathways and some of which may be bioactivation pathways [86]. An important example is the metabolism of the major tobacco carcinogen NNK. NNK can be metabolically activated by CYPs, but also reduced to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by CBR1 or 11-β-hydroxysteroid dehydrogenase type 1 (HSD11B1). Whereas the first phase I reaction produces the actual carcinogenic compounds, the latter facilitates the conjugation with glucuronic acid and thus excretion [89].

2.2.2 Carbonyl-metabolizing enzymes (CMEs)

The ubiquitous carbonyl moiety is often a determining factor for the biological effect of endogenous and exogenous compounds. For xenobiotic compounds, carbonyl reduction is usually regarded as a detoxification step as the resulting alcohol or hydroquinone is conjugated more easily [23, 33]. The term CMEs refers to enzymes.
involved in the NAD(P)^+/NAD(P)H-dependent oxidation or reduction of carbonyl compounds, i.e. aldehydes, ketones and quinones. Human CMEs include members of several enzyme (super)families including the family of alcohol dehydrogenases (ADHs) of the medium-chain dehydrogenases/reductases (MDR)-type, the aldehyde dehydrogenase (ALDH) superfamily, the NAD(P)H:quinone acceptor oxidoreductase family (NQO) and the superfamilies of short-chain dehydrogenases/reductases (SDR) and aldo-keto reductases (AKRs) [16]. Many of those enzymes exhibit endogenous functions as well as xenobiotic activities and, in the following, an emphasis will be on such with established xenobiotic activities [23, 33, 90-93].

2.2.2.1 Overall reactions in carbonyl metabolism and cofactor specificity

With NAD(H) as main cofactor, classical ADHs and ALDHs preferably participate in oxidative pathways, while AKRs, SDR and CRYZ, mostly relying on NADP(H), tend to perform reductive routes in carbonyl metabolism (Fig. 5) [16]. This is not without exceptions to the rule. For instance, the SDR member 15-(NAD^+)-hydroxyprostaglandin dehydrogenase (HPGD) is NAD^+-specific and several AKRs with dual cofactor specificity, albeit most often still with a preference for NADP(H), have been identified [94, 95]. NQO1 is unique in the aspect that it can use both NADH and NADPH with equal efficiency for two-electron reduction of quinones (Fig. 5). In contrast, NQO2 uses dihydronicotinamide riboside (NRH) rather than NAD(P)H [96].

2.2.2.2 The multiplicity of CMEs

Members of all of the above mentioned enzyme (super-)families have multiple functions in endogenous and xenobiotic metabolism. Human ADH1, ADH2 and ADH4 enzymes contribute to various extents to the detoxification of ethanol but are also involved in the metabolism of bile acids, steroids and retinoids [91, 97-99]. ADH3 (see 2.3) is the major formaldehyde scavenger, but also plays an important role in NO homeostasis by reducing GSNO [29-32, 100]. CRYZ, to date the best-studied human member of the MDR quinone oxidoreductase (QOR) family, displays enzymatic activity towards xenobiotic ortho-quinones, but has also been shown to bind to AU-rich elements in RNA, implying a potential role in post-transcriptional regulation [101-104]. On a side note, the family classification of CRYZ as provided by the Swiss-Prot database (http://ca.expasy.org/sprot/), where QORs including CRYZ are classified as a subfamily under the family of zinc-containing alcohol dehydrogenases, is partially
misleading. A complete screen of six completed eukaryotic genomes for MDR identified QORs as a discrete family belonging to the non-zinc containing MDR [103].

![Diagram of metabolic pathways](image)

Fig. 5. Types of reactions catalyzed by families of CMEs, modified from [16]. (A) Primary alcohols are oxidized to aldehydes mainly by ADHs of the MDR superfamily; the reverse reaction is often catalyzed by SDR or AKRs. Aldehydes are further oxidized to carboxylic acids by ALDHs. (B) Secondary alcohols are oxidized to ketones mainly by the action of ADH; again, the reverse reaction is often catalyzed by enzymes of the SDR or AKR superfamilies. (C) \( \zeta \)-Crystallin (CRYZ), belonging to the MDR family of QORs, and members of the NQO, SDR and AKR families catalyze the two-electron reduction of quinones to hydroquinones. The reverse reaction can be catalyzed by AKRs.

Endogenous substrates for ALDHs include intermediates in fatty acid metabolism (ALDH3 family, ALDH6A1), amino acid metabolism (ALDH4A1, ALDH5A1, ALDH6A1, ALDH7A1), the one-carbon pool by folate (ALDH1L1), retinoid metabolism (ALDH1, ALDH8A1), and the synthesis of the neurotransmitter \( \gamma \)-aminobutyric acid (ALDH9A1) [105-108]. Some ALDHs protect against aldehydes formed during lipid peroxidation, e.g. ALDH3A1 [109, 110]. A cetaldehyde (including after endogenous ADH-mediated oxidation of ethanol) is most efficiently oxidized by ALDH2 [105, 111]. Finally, ALDH1A1, ALDH2 and ALDH3A1 are involved in the metabolism of oxazaphosphorine anticancer chemotherapeutics as cyclophosphamide and ifosfamide [105, 112].

The SDR superfamily can functionally be subdivided into those involved in intermediary metabolism, e.g. UDP-galactose epimerase, dihydropteridin reductase, and sepiapterin reductase, and those involved in lipid hormone and mediator
metabolism including 3β-, 11β-, 17β-hydroxysteroid dehydrogenases, retinol-/retinal dehydrogenases and prostaglandin dehydrogenases [113]. Particularly the latter group contains many members with established additional functions in xenobiotic metabolism [23, 33, 90, 92, 114]. To name a few examples, the ubiquitous CBR1, probably the best-studied among the SDR, converts endogenous and exogenous substrates as diverse as prostaglandins, steroids, lipid aldehydes, NNK, anthracycline anticancer drugs and PAH-derived quinones [23]. Similarly, the microsomal enzyme HSD11B1 catalyzes the reversible reduction of 11-oxo glucocorticoids to 11-hydroxy glucocorticoids, but has also been demonstrated to metabolize NNK as well as cytotoxic quinones and the prospective anti-cancer drug oracin [115-117]. Finally, due to its function in the inactivation of prostaglandins, HPGD is considered to act as a tumor suppressor in many cancer types, but has also been suggested to play a role in the metabolism of PAH-derived quinones [114, 118, 119].

Although structurally unrelated, the AKRs exhibit very similar substrate specificities compared to the SDR including reduction of carbonyl functions in retinoids, steroids, eicosanoids, polyols, lipid peroxidation aldehydes and xenobiotics [23, 93]. Currently 13 human AKRs have been identified including ten cytosolic oxidoreductases, but also three potassium voltage-gated channel subunits (http://www.med.upenn.edu/akr/) [93]. Most AKRs have established functions in bioactivation/detoxification, as in the metabolism of anticancer drugs including anthracyclines (AKR1A1, AKR1C2) and oxazaphosphorines (AKR1B1) and of carcinogens including PAH metabolites (AKR1A1, AKR1C1-C4, AKR1B10), NNK (AKR1C1, C2, C4), and aflatoxin (AKR7A2, A3) [93, 120].

The NQO family (previously termed DT-diaphorases, currently also termed quinone reductases) consists of cytosolic FAD-containing enzymes that catalyze the reduction of cytotoxic quinones and nitroaromatic compounds. It comprises two human members, NQO1 and NQO2, with partly overlapping substrate specificities, but distinct cofactor requirement (as already mentioned under 2.2.2.1) [121]. NQO1 also plays a role in maintenance of the reduced form of the endogenous antioxidant coenzyme Q [122]. NQO-mediated reactions can also lead to metabolic activation of cytotoxic agents as e.g. the anti-tumor prodrug CB1954 [23, 121, 123, 124]. The latter property, in combination with multiple evidence that suggests NQOs as neoplastic markers, makes them interesting chemotherapeutic drug targets [124-126]. Moreover, recent evidence
suggests that NQOs are involved in the control of proteasomal degradation of tumor suppressors including p53 [127, 128].

In summary, CMEs include several evolutionarily unrelated enzyme (super)families with central and often competing or overlapping functions in the metabolism of steroids, bile acids, prostaglandins and retinoids, as well as in the biotransformation of xenobiotic carbonyl compounds including e.g. ethanol, formaldehyde, NNK, PAH-derived metabolites and different groups of anti-cancer therapeutics. Hence, CMEs are implicated in carcinogenesis as well as cancer chemotherapeutic drug resistance. Interestingly, recent evidence moreover suggests non-oxidoreductase functions for some quinone reductases, e.g. CRYZ as regulator of RNA stability and NQOs as modulators of proteasomal tumor suppressor degradation [102, 104, 127, 128].

![Chemical structures](image)

Fig. 6: Chemical structures of the substrates menadione (A) and 9,10-PQ (B) as well as the inhibitors dicoumarol (C) and rutin (D). Rutin is the glycoside between the flavonoid quercetin and the disaccharide rutinose.

As a consequence of the overlapping substrate specificities, it is a challenge to assign specific enzymes to reductive biotransformation reactions mediated by CMEs in crude protein mixtures. Under circumstances of limited material, e.g. when using cultured normal cells with a finite life span, a virtually collective assessment of CME-mediated xenobiotic metabolism (with the exception of ALDHs and classical ADHs) is possible by the use of two promiscuous substrate quinones, menadione and 9,10-phenanthrenequinone (9,10-PQ) in combination with NAD(P)H; notably, those two quinones are often among the best substrates for CMEs with quinone reduction activity [33, 84, 101, 104, 129-132]. Furthermore, the assessment of cofactor specificity and the use of relevant inhibitors, e.g. dicoumarol and rutin, can allow for some discrimination between different CME activities [18, 129]. Dicoumarol inhibits NQO1, CBRs and
CRY Z, while rutin and related flavonoids inhibit CBRs, some AKRs and NQO2 [101, 132-135]. The structures of menadione, 9,10-PQ, dicoumarol and rutin are shown above (Fig. 6).

2.2.3 Glutathione (GSH) - small but powerful

GSH, the tripeptide γ-glutamylcysteinylglycine (Fig. 7), is found in millimolar concentrations in most cells and is one of the most important low molecular weight compounds in the defense against reactive metabolites and xenobiotics [86, 136]. The reactive cysteine thiol group in GSH forms the basis of its cytoprotective function. Scavenging of radicals and reactive metabolites by GSH often occurs spontaneously by donation of the thiol hydrogen or proton, respectively, most commonly followed by the reaction with another GSH molecule to form glutathione disulfide (GSSG). If a peroxide is the reactive metabolite, this reaction is catalyzed by the enzyme glutathione peroxidase. Under normal redox conditions, GSSG is recycled to GSH by GSSG reductase under concomitant oxidation of NADPH. Under conditions of oxidative stress, however, reactive oxygen species accumulate and GSH and NADPH are depleted, making the cell more vulnerable to toxic challenge. GSH depletion contributes to the pathogenesis of a number of disease conditions including cancer, neurodegenerative and cardiovascular diseases [137, 138].

2.2.4 Glutathione transferases (GSTs)

In some cases, the formation of GSH conjugates can occur spontaneously, e.g. the GSH adduct of formaldehyde, S-hydroxymethylglutathione (HMGSH, an important intermediate in formaldehyde detoxification), is formed non-enzymatically [86]. Yet for the most part, GSTs catalyze the nucleophilic attack of GSH on electrophilic atoms of hydrophobic xenobiotic compounds and lipid peroxidation products and thus contribute to the detoxification of numerous carcinogenic, mutagenic and toxic compounds [139]. There are two major types of mammalian GSTs, the soluble GSTs which belong to the multi-gene GST superfamily and the microsomal GSTs which
belong to the MAPEG (Membrane-Associated Proteins in Eicosanoid and Glutathione metabolism) family [140]. Their catalytic activity relies on the ability to bring GSH in proximity and correct orientation in respect to the hydrophobic substrate as well as to lower the pK_a of the GSH thiol group when it is bound to the active site (from \(\approx 9\) in solution to \(\approx 6-7\) upon binding). Hence, at physiological pH, GST-bound GSH tends to occur in the more reactive thiolate anion form (\(\text{GS}^-\)) [141, 142]. Glutathionesulfonic acid, occurring as the sulfonate at physiological pH in solution, is a potent and in respect to GSH competitive inhibitor of GSTs [143, 144]. GSNO appears to be able to modulate GST activity by two mechanisms, competitive inhibition and covalent modification, presumably by S-nitrosation of a cysteine side chain [145].

2.2.5 Expression of xenobiotic enzymes in buccal keratinocytes

Previous studies have assessed expression of CYPs, GSTs and other phase II biotransformation enzymes in NBK, SVpgC2a and SqCC/Y1. Overall, these studies suggested that NBK, similar to normal tissue specimens, expressed CY P1A1, CY P1A2, CY P2C, CY P2D6, CY P2E1 and CY P3A, albeit for the most part at levels close to detection limit [146, 147]. Phase II enzymes were more abundantly expressed, particularly sulfotransferase 1A3, microsomal GST and GST P1. CY P3A was lost in the immortalized buccal cell line SVpgC2a and CY P2C, CY P2D6 and CY P3A 4/7 were lost in SqCC/Y 1. In contrast, CY P1A1 and CY P1B1 were elevated in SV pgC2a [147].

Furthermore, early generation microarray analysis was applied to investigate CME transcription in cultured normal and transformed buccal keratinocytes [148]. This study indicated expression of ADHs, ALDHs, SDR and AKRs including such with established functions in xenobiotic metabolism, e.g. ADH3, CRY Z, ALDH2, CBR1 and AKR1A1. Expression of several of the detected CME transcripts was altered in the transformed cell lines. Expression of ADH3 was assessed on transcript, protein and activity level in oral tissue, NBK, SVpgC2a and SqCC/Y 1 including in organotypic culture [27, 149]. These studies revealed that ADH3 mRNA is confined to the basal and parabasal layers, while the ADH3 protein was detected at similar levels throughout the epithelia, including those generated in vitro. This suggested that ADH3 transcription might be regulated in association with proliferation or TSD while the protein is sufficiently stable to last throughout tissue turnover.
2.3 ALCOHOL DEHYDROGENASE 3

The highly conserved and ubiquitous ADH3 enzyme is considered to represent the ancestral form of the MDR-type ADHs [150-155]. Similar to many other CMEs, ADH3 exhibits activity towards endogenous as well as exogenous compounds, of which the most important appear to be HMGSH and GSNO. By catalyzing the oxidation of HMGSH, the spontaneous GSH conjugate of formaldehyde, ADH3 is central to the detoxification of endogenous and exogenous formaldehyde, including in the buccal mucosa [27, 91, 100]. In addition, during the last decade evidence has accumulated that strongly suggests that ADH3, through the irreversible GSNO reductase activity, affects the transnitrosation equilibrium between GSNO and S-nitrosated proteins and thus plays an important role in endogenous NO homeostasis [29, 30, 32]. Other functions attributed to ADH3 include first-pass ethanol metabolism, contribution to retinoic acid formation and oxidation of $\omega$-hydroxy fatty acids [26, 156-158].

2.3.1 ADH3 - a protein of many names

Nomenclature of the ADHs, a family comprised of several classes including different isozymes, is still a subject under debate (http://ca.expasy.org/sprot/) [159, 160]. In line with ADH family classification, ADH3 is frequently termed “class III alcohol dehydrogenase” in the literature. As most mammalian ADHs are dimeric molecules, they have also often been named according to their individual subunits, facilitating the distinction between homo- and heterodimeric enzymes. In this context, ADH3 is referred to as “$\gamma\gamma$ ADH”. Based on its catalytic activities, ADH3 has been called “glutathione-dependent formaldehyde dehydrogenase” and “GSNO reductase” (GSNOR) [32, 161]. Finally, further complexity is added by the fact that the gene nomenclature was not updated in parallel with the protein nomenclature and the gene coding for human ADH3 is currently still named ADH5 according to the Swiss-Prot database (http://ca.expasy.org/sprot/).

2.3.2 Structure/function relationships

ADH3 exhibits a homodimeric structure with two zinc atoms per 40 kDa subunit, each comprised of two domains, designated the catalytic and the coenzyme binding domain (Fig. 8). While one of the zinc atoms is considered to serve a structural function only, the other zinc atom functions as a Lewis acid and activates the substrate in the
active site which is located in a cleft between the catalytic domain and the coenzyme binding domain [162]. These overall structural properties are shared by all classical mammalian ADH structures. However, relative to other ADHs, the structure of ADH3 shows distinct differences in the immediate vicinity of and within the active site and moreover displays a semi-open domain conformation, features that are reflected in substrate specificity and kinetic properties. More precisely, residues 55-61 and 112-120 adopt a different secondary structure and move away from the catalytic cleft, which leads to an enlargement of the active site and generates a broader entrance to the substrate-binding pocket than in the ADH1 enzymes [162]. Hence the active site cannot be saturated with ethanol, but accommodates larger substrates such as HMGSH, GSNO, medium-chain alcohols, aldehydes and α-hydroxy fatty acids [26, 91, 150, 163]. Furthermore, several ADH3-specific residues within the active site play critical roles in ligand binding. The efficient binding of HMGSH (see Fig. 9 for the chemical structure) is mainly mediated by R114, interacting with the glycyl branch carboxyl group, the residues D55 and E57, interacting with the amino group of the γ-glutamyl branch, as well as the active site zinc interacting with the hydroxyl group (Fig. 8) [162, 164, 165].

*Fig. 8: Left: The dimeric structure of ADH3, showing the different monomers (dark-gray and light-gray) and the position of zinc atoms (black), NAD (dark-gray) and HMGSH (black) in the light-gray monomer. Right: Close-up view of the active site with NAD and HMGSH, demonstrating orientation of the substrate between the active-site zinc and the anion-binding pocket, represented by R114. Amino acids interacting electrostatically with HMGSH, i.e. D55, E57 and R114, as well as both zinc atoms are displayed. Only the active site zinc ion is labeled Zn. The figure was generated from pdb-file 1MC5 using Molsoft ICM-Browser [165].*
Tertiary structure differences between the apoenzyme, binary and ternary complexes are consistent with a random bi-bi kinetic mechanism observed for oxidation of HMGSH and 12-hydroxydodecanoic acid under concomitant conversion of NAD⁺ [163, 165-167]. Binding of the substrate or NAD(H) does not entail significant domain movement as observed upon coenzyme binding in other ADHs where an ordered bi-bi kinetic mechanism is predominant. In contrast, domain closure is executed after ternary complex formation which allows random addition of substrate and coenzyme in the course of catalysis [167].

2.3.3 Formaldehyde detoxification

Formaldehyde, the simplest carbonyl compound, has recently been classified as a human carcinogen [24]. Owing to the polarized carbonyl group, formaldehyde can be attacked by nucleophiles and electrophiles and, hence, participate in various substitution and addition reactions. A cellular environment holds various potential reactants for formaldehyde: For instance, thiols readily form hemithioacetals with formaldehyde as e.g. in the reaction with GSH yielding HMGSH, which can be regarded as a first-line defense against formaldehyde toxicity. In contrast, the reaction with amines results in Schiff bases which initiates the formation of DNA-DNA, DNA-protein and protein-protein crosslinks where amino groups present in DNA and proteins are stably connected via methylene bridges derived from formaldehyde [168]. The high DNA reactivity manifests itself in mutagenic effects and chromosomal changes which, in combination with formaldehyde-associated activation of proliferation, are considered to underlie formaldehyde carcinogenicity [24, 169]. Moreover, formaldehyde is a potent respiratory irritant with well-established bronchoconstricting and asthma-exacerbating effects; the underlying molecular causes, however, are poorly understood [170-173].

Formaldehyde is ubiquitous in our environment, as for instance released from plywood, present in tobacco smoke or in car exhaust. Moreover, certain occupational groups including workers in wood industry, garment industry, pathologists and embalmers can be particularly exposed to formaldehyde [24, 170]. Formaldehyde can also be formed endogenously as a result of normal metabolic pathways (e.g. in serine and glycine metabolism) or in the course of biotransformation processes, as for
example by oxidative demethylation of xenobiotics by CYPs or oxidation of ingested methanol [174-178].

In humans, formaldehyde can be directly metabolized by some ALDHs, but these enzymes exhibit Michaelis constants for free formaldehyde that are by several magnitudes higher than the one ADH3 exhibits for HMGSH [179-181]. Under typical cytosolic redox conditions, i.e. with GSH present in millimolar concentrations and a high \( \text{NAD}^+ / \text{NADH} \) ratio, ADH3 is certainly the most efficient formaldehyde-metabolizing enzyme and thus, together with esterase D (ESTD, also termed S-formylglutathione hydrolase) constitutes the primary defense against formaldehyde damage (Fig. 9) [182]. Several functional studies uniformly support the importance of ADH3 for formaldehyde resistance in prokaryotic as well as in eukaryotic organisms: Formaldehyde-resistance in an E. coli strain is mediated by a plasmid carrying the gene for an ADH3-homologue [183, 184]. Overexpression of Arabidopsis thaliana ADH3 in yeast and plants confers improved resistance towards exogenously added formaldehyde [185]. Finally, the LD\(_{50}\) for formaldehyde in Adh3-deficient mice is significantly lower than the one in wild-type mice [100].

Fig. 9: ADH3-mediated formaldehyde detoxification pathway. (1) Formaldehyde is non-enzymatically conjugated with GSH to yield HMGSH, which (2) is oxidized by ADH3 under concomitant conversion of \( \text{NAD}^+ \) to give S-formylglutathione. This step is followed by (3) ESTD-mediated hydrolysis which regenerates GSH and yields formate.
2.3.4 Reduction of S-nitrosoglutathione

2.3.4.1 Role in nitric oxide (NO) signaling

NO signaling is involved in a large array of physiological functions including vasodilation, cell death, regulation of redox potential and control of respiration [186]. NO is formed endogenously by the action of several NO synthases which convert the amino acid arginine to citrulline and NO (Fig. 10). With a biological half-life of only a few seconds, NO is relatively short-lived and NO bioactivity is to a large part conveyed by post-translational modification of proteins, by metal nitrosation or S-nitrosation [186, 187]. Hence, intracellular NO signaling is in part mediated through protein S-nitrosation with currently more than 100 identified potential targets, including proteins involved in cellular processes as diverse as apoptosis, membrane trafficking and iron homeostasis [186].

![Fig. 10: Nitric oxide synthases convert arginine to citrulline and NO under concomitant consumption of NADPH and O₂. Then, NO can modify proteins by covalently binding to metal centers (metal nitrosation) or cysteine residues (S-nitrosation).](image)

![Fig. 11: Structure of GSNO.](image)
S-nitrosated proteins appear to exist in transnitrosation equilibrium with GSNO, the most common low molecular weight S-nitrosothiol (SNO) (Fig. 11) [188]. During the last years, evidence has emerged that supports the concept that this equilibrium is affected by ADH3 through its GSNO reductase activity (Fig. 12) [30, 32]. Notably, the ADH3-mediated GSNO reductase activity is the only GSNO-converting activity detected so far that does not result in NO release, in contrast to glutathione peroxidase, the thioredoxin system, xanthine oxidase and Cu/Zn superoxide dismutase [28, 29, 189-192]. Instead, the NO moiety is reduced and irreversibly removed from the NO pool, compatible with an NO terminase function [28, 29]. Furthermore, it is by magnitudes the most efficient GSNO-converting activity among all suggested activities, provided the appropriate cofactor NADH [28, 29, 189-192]. Also functional studies support this concept: In cultured cells, the degree of downregulation of ADH3 by RNA interference correlated inversely with SNO levels [30]. Moreover, Adh3-deficient mice show significantly increased SNO levels in lung homogenates [32]. Thus, in its function as GSNO reductase, ADH3 appears to indirectly govern levels of protein SNOs and is therefore likely to serve an important regulatory function in NO signaling.

\[
\text{Protein(Cys)-S-N=O + GSH} \leftrightarrow \text{Protein(Cys)-SH + GS-N=O}
\]

\[
\text{NADH} \quad \text{ADH3} \quad \text{NAD}^+ \quad \text{GS-NH}_2\text{OH}
\]

Fig. 12: The transnitrosation equilibrium between protein SNOs and GSNO is shifted towards the right-hand side by ADH3-mediated GSNO reduction under concomitant conversion of NADH.

Deregulated levels of cellular SNOs are often associated with medical conditions including cerebral ischemia, Alzheimer’s disease, sclerosis, and asthma [32, 193-196]. Notably, genetic and functional evidence support the involvement of ADH3 in asthma. GSNO functions as an endogenous bronchodilator in airway lining fluid and performs as such a protective role in asthma [197]. In wild-type mice exhibiting airway hyperresponsivity, allergen challenge results in increased GSNO reductase activity in airway lining fluid and SNO depletion in adjacent lung cells. In contrast, Adh3-deficient mice are protected from airway hyperresponsivity [32]. In addition, two single
nucleotide polymorphisms in the gene coding for ADH3 have been coupled to asthma susceptibility [161].

2.3.4.2 Product formation following GSNO reduction

ADH3-mediated GSNO reduction results in a transient semimercaptal, S-hydroxylaminoglutathione (cf. Fig. 12), but conclusions from the literature have diverged about the next step. The spontaneous rearrangement to glutathione sulfinamide or the reaction with GSH to form GSSG as the final product have been suggested [28, 29, 31]. In addition, different reaction mechanisms with GSH have been put forward. One study provided evidence for equimolar consumption of NADH and GSNO as well as the direct interception of the intermediate by GSH to yield GSSG and hydroxylamine [28]. In contrast, another study suggested the reduction of S-hydroxylaminoglutathione to S-aminoglutathione under concomitant oxidation of a second NADH molecule, followed by the reaction with GSH to yield GSSG and ammonia [31].
3 AIMS OF THIS THESIS

The aims of this thesis were two-fold. The first overall aim was to contribute to the elucidation of buccal carcinogenesis mechanisms and to discover novel multiple tumor markers and drug targets with the long-term goal to propose novel diagnostic and therapeutic strategies for BSCC. These questions were addressed in the described in vitro model for buccal carcinogenesis including proliferative and differentiated states. Here, the specific aims were:

- to assess overall differential gene expression in the two transformed buccal keratinocyte lines SVpgC2a and SqCC/Y1 relative to NBK
- to test a combination of bioinformatics tools for the integration of gene expression data including the detection of tumor suppressor/promoter genes as well as novel multiple biomarkers including molecular networks and gene ontology (GO) categories
- to establish protocols for the selective culture of proliferative and variably differentiated NBK in order to mimic the process of buccal TSD in culture and assess whether ADH3 transcription associates with proliferation or TSD
- to analyze expression and activity of CMEs in SVpgC2a and SqCC/Y1 relative to NBK as well as in association to the process of buccal TSD

The second overall aim was to assess the major buccal formaldehyde scavenger ADH3 in terms of exogenous/endogenous substrate competition, i.e. in its dual function as formaldehyde dehydrogenase and GSNO reductase. Here, the specific aims were:

- to assess how formaldehyde affects ADH3-mediated GSNO reduction
- to study product formation following GSNO reduction as a function of GSH concentrations
- to test the products of ADH3-mediated GSNO reduction as GST inhibitors
- to examine inhibition of ADH3-mediated GSNO reduction by different substrate analogues
4 EXPERIMENTAL PROCEDURES

The following constitutes a short summary of the various methods used in the present thesis work. For more detailed descriptions, I would like to refer to the attached original papers I-V and references therein.

Cell culture of NBK, SVpgC2a and SqCC/Y 1 was in a serum-free medium with high levels of amino acids (EMHA, Papers I-IV). Post-confluent culture in absence or presence of FBS was used for induction of TSD (Papers II and III). The colony-forming efficiency (CFE) assay was used to assess proliferative ability of NBK (Paper II). Formaldehyde exposure was in a chemically defined variant of EMHA and followed by the determination of intracellular SNOs by a photometric assay involving Cu\(^{2+}\)-triggered release of NO and subsequent diazotization and derivatization with sulfanilamid and N-(1-naphthyl)ethylenediamine (Saville-Griess assay, Paper IV).

Assessment of gene expression at transcript level was performed by DNA microarray analysis (Papers I and III) or standardized reverse transcriptase-polymerase chain reaction (StaRT-PCR, Paper II). Assessment at protein level was performed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), subsequent in-gel digestion and matrix-assisted laser/desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) followed by mass fingerprinting (Paper I). For some low-abundant proteins also Western Blot analysis was performed.

Bioinformatics processing of large-scale gene expression data included the sequential use of three bioinformatics tools, the Gene Ontology Tree Machine (GOTM), AffyAnnotator, and Ingenuity Pathway Analysis (IPA, Paper I). The GO is a controlled vocabulary, developed and maintained by the Gene Ontology Consortium (http://www.geneontology.org/) that sorts gene products into categories according to their associated biological processes, molecular functions and cellular components. The web-based tool GOTM uses a statistical hypergeometric test to analyze for enrichment of such categories in any interesting data set (http://bioinfo.vanderbilt.edu/gotm/). AffyAnnotator, developed at the Bioinformatics and Expression Analysis Core facility at Karolinska Institutet, allows for visualization and sorting of transcripts on Affymetrix chips according to the GO (http://www.bea.ki.se/jnlp/). IPA can be applied
to search for molecular networks and pathways that are common to any list of interesting genes. IPA relies on a large knowledge database consisting of information about interactions between gene products which has been extracted from the literature (IPA 4.0, Ingenuity Systems, http://www.ingenuity.com).

Synthesis of substrates and inhibitors: GSNO was synthesized according to Hart [198] (Papers IV and V). Glutathione sulfanamide and sulfinic acid were obtained following ADH3-mediated reduction of GSNO and separation of the products by anion exchange chromatography. Product fractions were identified by electrospray ionization mass spectrometry (ESI MS, Paper IV). S-acetamidoglutathione was synthesized by acetamidylaion of GSH with iodoacetamide, resolved on an anion exchange column, and its identity was verified by ESI MS (Paper V). Concentrations of glutathione sulfanamide, sulfinic acid and S-acetamidoglutathione were determined by ninhydrin-based amino acid analysis (Papers IV and V).

Enzymatic activity for dehydrogenase/reductase reactions was mostly assessed spectrophotometrically by following the concomitant conversion of NAD(P)(H) and/or GSNO at 340 nm (Papers III-V). In paper IV, also fluorescence spectrophotometry ($\lambda_{exc} = 340$ nm, $\lambda_{em} = 455$ nm) was used for this purpose. Microsomal glutathione transferase 1 (MGST1) activity was monitored by following the formation of the GSH-dinitrobenzene conjugate from GSH and 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm. Protein-ligand docking simulations were performed with ICM 3.5 (Molsoft LLC) (Paper V) [199-201].

Quantitative product analysis of GSNO reduction was performed by resolving the reaction mixtures on a strong anion exchange column followed by peak integration. Peak intensities were calibrated for commercially available reactants and products. Identities of reactant/product fractions were verified by ESI MS. Hydroxylamine concentrations were determined with a modified form of a colorimetric assay where hydroxylamine is trapped as a quinoline oxime [202].
5 RESULTS AND DISCUSSION

5.1 DIFFERENTIAL GENE EXPRESSION IN TRANSFORMED BUCCAL KERATINOCYTES RELATIVE TO NBK

5.1.1 Differentially expressed proteins are confirmative of the model system and include potential novel tumor markers (Paper I)

Protein profiling by 2D-PAGE, subsequent in gel digestion and MALDI-TOF MS for mass fingerprinting led to the identification of 19 differentially expressed proteins including several isoelectric variants in one or both of the transformed cell lines SVpgC2a and SqCC/Y1. In agreement with decrease or loss of CKs as well-established characteristics of oral SCC, CK5, CK14, CK17 and CK6 were essentially lost in SVpgC2a and CK7 was significantly decreased in SVpgC2a and SqCC/Y1 [39, 203, 204]. In contrast, the heat shock proteins (HSPs) HSP27, HSP60 and HSP70 were upregulated in one or both of the transformed cell lines. All three HSPs have been found overexpressed in oral premalignant lesions and carcinomas and have thus previously been considered as diagnostic markers [61, 205-207]. Notably, novel aspects in that context were the observed selective down- and upregulation of distinct isoelectric variants of CK5 and HSP60, respectively, in the BSCC cell line. Additional proteins implicated from this study previously considered as buccal or oral tumor markers included heterogeneous ribonucleoprotein (hnRNP) B1, stratifin (SFN) and α-enolase [208-210]. In oral SCC, SFN, an epithelium-specific p53-transactivated tumor suppressor gene, can be inactivated by either loss of p53 function or promoter methylation and loss of SFN expression appears to occur early in carcinogenesis [209, 211]. In contrast, a study on differential protein expression in BSCC tissue specimens demonstrated strong upregulation of SFN, which, however, might reflect the specified tissue heterogeneity rather than tumor-associated changes [210]. Notably, the remaining altered proteins, namely acetyl-CoA acetyltransferase (ACAT1), elongation factor-Tu, ferritin light chain, fumarate hydratase, glutamate dehydrogenase, hnRNP C1/C2, peroxiredoxin 3 and tropomyosin 3 had not been implicated in previous studies on buccal or oral SCC and thus constituted potential novel markers of BSCC.
5.1.2 Bioinformatics processing identifies further multiple and integrative biomarkers for BSCC (Paper I)

Of the discussed differentially expressed proteins identified by 2D PAGE expression only some were already altered at the transcript level including all CKs, SFN and ACAT1. Otherwise, changes in protein and transcript levels for individual genes were mostly inconsistent. For HSP27 and HSP70 changes in transcript and protein levels even anti-correlated, possibly reflecting autoregulation of HSP transcription by abundant HSPs [212]. Generally, transcript assessment is considered to capture only about 40% of the changes in protein expression [213]. Hence, the results confirm that transcript data should be interpreted with caution in tumor marker studies when focused on single gene markers. However, differential gene expression on mRNA level often reflects protein abundance better in functional categories than on single gene level [214]. Accordingly, functional classification by bioinformatics tools can allow for bridging the gap between transcript and protein data.

Fig. 13: Scheme of overall experimental approach and data interpretation. NBK, SVpgC2a and SqCC/Y1 were cultured under a standardized serum-free condition and differential expression was assessed by 2D-PAGE and DNA microarray analysis. A sequence of three bioinformatics tools (GOTM, AffyAnnotator and IPA, indicated by dashed lines) was used to analyze the data sets. Finally, expression of some resulting key regulators was assessed by Western blot analysis.
Here, a strategy using three bioinformatics tools was devised (Fig. 13): The differential protein signatures of each cell line relative to NBK were used for GOTM-based enrichment for functional and structural categories which yielded eight and four GO categories for SVpgC2a and SqCC/Y1, respectively (shown for SqCC/Y1 in Fig. 14). Target genes within the resultant GO categories were then selected from the transcript data by AffyAnnotator. A representative AffyAnnotator-derived heat map shows the results for transcripts within the GO category response to protein stimulus in SqCC/Y1, including the six transcripts thereof selected for further analysis (Fig. 15). Finally, within the pooled transcript data from all enriched GO categories for each cell line, IPA identified aberrant molecular networks and their key regulator genes. The altered transcripts in SVpgC2a and SqCC/Y1 generated 10 and 6 highly significant molecular networks, respectively (see Fig. 16 for an example). For a majority of the networks in both cell lines, IPA suggested “cancer”, “cell death” or “cellular growth and proliferation” as one of the associated top three functions.

Next, screening of these molecular networks for key regulator genes exhibiting at least three interactions with altered transcripts resulted in the identification of 18 potential tumor suppressor/promoter genes. Most hereby identified transcription factors are known to exhibit multiple roles in cancer causation. The key regulator genes implicated with both lines, CDKN2A, TP53, MYC, MYCN and SP1 represented genes and markers for involvement in various oral cancers, including BSCC [4, 61, 215-218]. Eight further genes identified from one of the transformed cell lines, TP73L, SMAD4, RELA, PPARG, ARNT2, EPAS1, HIF1A and SP3 have previously been shown to directly or indirectly associate to oral or buccal carcinoma [4, 61, 62, 217-222]. In contrast, deregulation of CEBPA, HNF4A, SIM1, NFYB and MYOD1 or related proteins/functions was not previously implicated for buccal/oral cancer and thus, those genes constitute potential novel key regulator genes for buccal carcinogenesis.

Five of these 18 transcription factors indicated as deregulated through IPA were subjected to Western blot analysis. Relative to NBK, expression of transcription factors Sp1 (SP1), Sp3 (SP3) and c-Myc proto-oncogene (MYC) was increased in both SVpgC2a and SqCC/Y1. Differently, hypoxia-inducible factor 1α (HIF1A), implied from IPA as deregulated in SVpgC2a, was increased selectively in SqCC/Y1 and p16 (CDKN2A), implied from IPA for both transformed cell lines, was increased selectively in SVpgC2a. Although in part seemingly contradictory, this might actually reflect the
fact that aberrant key regulator function only variably is a result of altered transcript or protein levels, but also involves other mechanisms as e.g. post-translational modifications or inhibition of nuclear translocation [223]. Notably, changes in transcript and protein levels were mostly inconsistent also for these five key regulator genes; qualitative agreement was only observed for transcript and protein levels of p16.

![Diagram](image)

**Fig. 14:** GOTM data output exemplified for SqCC/Y1 relative to NBK. A total of seven enriched GO categories were identified (displayed in red) and the four at the highest hierarchical level (depicted with arrows) were used in further analysis.

In summary, the described approach led to the identification of multiple potential markers for BSCC, both at transcript and at protein level, as well as of several potential integrative biomarkers such as GO categories and deregulated molecular networks including their aberrant key regulator genes. Expression changes in a relatively low number of abundant proteins, combined with the use of bioinformatics tools and the inclusion of large-scale transcript data for which little material is required, ultimately allowed for identification of low-abundance transcription factors. Hence, the methodology seems useful for the analysis of gene expression data under circumstances of limited samples, e.g. in personalized medicine.
Fig. 15: AffyAnnotator-derived heat map for the GO category response to protein stimulus, enriched in SqCC/Y1 relative to NBK (see Fig. 14). Four pair-wise comparisons are shown. Significant Fold Changes are visualized with plus or minus signs and transcripts significantly deregulated in all four comparisons (marked with an asterisk) were chosen for further analysis. Red or green color, respectively, indicates increased or decreased transcripts.

Fig. 16: Example of a molecular network generated by IPA from altered transcripts within enriched GO categories for SVpgC2a relative to NBK. Here, TP53 and TP73L are central key regulators. Red or green color, respectively, indicates increased or decreased transcripts.
5.1.3 SVpgC2a and SqCC/Y1 exhibit altered quinone reduction potential (Paper III)

CME gene transcription was assessed in the transformed cell lines relative to NBK and a total of 8 CME genes were shown to be differentially transcribed in SVpgC2a and/or SqCC/Y1. Transcripts of CRYZ, ALDH1A3, ALDH2, ALDH4A1, GALE, HSD17B2 and QDPR were decreased in one or both of the transformed cell lines and transcripts of ALDH3B1 were increased in both. These results were in very good agreement with an earlier generation microarray analysis [148]. CME-mediated quinone reduction activity in crude cell lysates of NBK, SVpgC2a and SqCC/Y1 had not been studied previously.

As to SVpgC2a, overall menadione reduction activity was unchanged relative to NBK, but not inhibited by dicoumarol, excluding CBR1 or NQO1 as significant contributors to that activity [132, 134]. At the same time, rutin-inhibitable 9,10-PQ reduction was upregulated, which suggests increased AKR activity [135]. Thus, the immortalized cell line SVpgC2a exhibited altered competence for quinone reduction relative to NBK, both in terms of specificity and potency. Differently, in SqCC/Y1 relative to NBK, NQO1-like (i.e. dicoumarol-sensitive and characterized by dual cofactor specificity) was upregulated and overall 9,10-PQ reduction activity increased, but, in contrast to NBK, with unaltered rutin-inhibitable fraction. The underlying experimental design does not allow for specific enzyme assignments of any of these activities, but nevertheless the results imply that quinone reduction competence is altered in association with cell transformation. The possible implications for buccal drug response and for the use of continuous cell lines in toxicity testing emphasize the need for further studies.

5.2 DIFFERENTIAL GENE EXPRESSION IN PROLIFERATIVE AND DIFFERENTIATED BUCCAL KERATINOCYTES

5.2.1 Confluency with or without FBS induces TSD in buccal keratinocytes (Papers II and III)

In paper II, different culture protocols were used to induce TSD in NBK. The course of this process was monitored by assessment of morphological changes under the microscope, assessment of proliferative ability by use of the CFE assay, and finally by
assessment of established markers of proliferation and TSD. Maintaining NBK at confluency for five days induced a squamous morphology, inhibited CFE, decreased transcription of two proliferation markers, cell division control protein 2 (CDC2) and proliferating cell nuclear antigen (PCNA), and increased transcription of two markers of TSD, involucrin (IVL) and small proline-rich protein (SPR) 1. Addition of 2 % and 10 % FBS variably influenced these effects: First, inhibition of CFE and loss of CDC2 and PCNA transcription was partly counteracted. Second, IVL, but not SPR1 transcription, was promoted. Third, transcription of CK5, a marker for basal keratinocytes, was only decreased in FBS-containing post-confluent cultures. After that, continued culture for ten and 15 consecutive days gradually abolished CFE in absence and presence of FBS, but only few additional transcriptional changes were observed, including the loss of CK5 also in post-confluent cultures without FBS and a strong increase in IVL transcription in the presence of 2 % FBS.

Fig. 17: Morphologies of NBK grown to a confluent state in serum-free medium (A) and maintained at confluency for three days with 5 % FBS (B).

Paper III aimed at assessing early changes in TSD. Thus, a culture protocol including 5 % FBS was applied in order to enrich for a proliferative keratinocyte phenotype at the onset of TSD. Cells were collected when they exhibited the typical flattened squamous morphology associated with the onset of TSD which usually was after three days post-confluent culture (Fig. 17). Markers of proliferation were largely unaffected under this growth protocol, while markers of early TSD (IVL and SPR3, gene name SPRR3) and one marker of suprabasal keratinocytes (CK13, gene name KRT13) were drastically increased (Fig. 18). Expression of a marker of late TSD (TGM1) and another marker of suprabasal keratinocytes (CK4, gene name KRT4) was less promoted, with a trend to increased levels that failed significance (Fig. 18). Here,
in order to distinguish between TSD-associated and serum-related effects, SVpgC2a and SqCC/Y1, both TSD-deficient, were subjected to the same growth protocol. As expected, those cell lines failed to regulate all markers of TSD in response to serum (Fig. 18). The overall results demonstrate that different culture protocols can be used in efforts to selectively enrich proliferative and differentiated keratinocyte phenotypes, i.e. different strata in a normal buccal mucosa.

5.2.2 ADH3 transcription associates with proliferation (Paper II)

Several growth protocols involving post-confluent culture for up to 15 days with and without FBS were used to study the association of ADH3 transcription with markers of proliferation and/or TSD. Transcript levels were assessed by StaRT-PCR technique. Loss of transcripts for ADH3 in post-confluent cultures showed good positive correlation with loss of proliferation markers, while neither a positive correlation with CK5 nor a negative correlation with the TSD markers was evident. Hence, ADH3 transcription associated with proliferation and could thus serve as a proliferation marker.

In this context it should be emphasized that only ADH3 mRNA levels associate with proliferation. In contrast, it has been demonstrated that the ADH3 protein is not confined to (para-)basal layers, but maintained in active form throughout the entire keratinocyte life span [27]. This might simply constitute an energy-saving mechanism where unnecessary mRNA synthesis is avoided once the protein is abundant and stable. Additionally, preserving actively formaldehyde-scavenging ADH3 throughout the epithelium, while confining transcription to the basal tissue compartment, the least affected by formaldehyde and other genotoxic agents, could also be a mechanism to assure correct transcripts in the proliferative cells.

5.2.3 Transcription of at least three CMEs is altered at the onset of TSD (Paper III)

Using DNA microarray technique, the expression of genes coding for CMEs was assessed in NBK, SVpgC2a and SqCC/Y1 before and after three days of post-confluent culture with 5 % FBS. All 58 members of the ADH, ALDH, SDR, AKR and NQO (super-)families present on the Human Genome Focus Chip (contains 8400 genes) were evaluated in this study. Serum exposure for NBK resulted in more than two-fold altered expression of five CMEs, including increased expression of ALDH1A3, DHRS3,
HPGD, and AKR1A1, and decreased expression of ALDH4A1 (Fig. 18). The transcriptional increase in response to serum was in part conserved in one or both of the transformed cell lines for ALDH1A3 (4.4-fold increase in SV pgC2a) and DHRS3 (2.8-fold increase in both SV pgC2a and SQCC/Y1). The fact that the addition of serum causes these genes to change expression substantially in SV pgC2a and SQCC/Y1 without causing them to differentiate highlights the possibility that these changes could be due to TSD-unrelated effects of serum. Hence, in line with the observed pattern for TSD markers, CME genes associated with serum-induced growth inhibition/TSD should typically be represented by transcriptional changes, which are observed in NBK upon serum exposure, but not in SV pgC2a and SQCC/Y1.

**Fig. 18:** Serum-responsive gene expression of suprabasal keratinocyte markers (KRT4, KRT13), markers of early (IVL, SPRR3) and late TSD (TGM1), proliferation markers (PCNA, CDC2) and significantly altered CMEs (ALDH1A3, ALDH4A1, DHRS3, HPGD, and AKR1A1). Dark-gray bars depict results for confluent cells; light-gray bars for post-confluent serum-exposed cells. Average signal intensities (y-values) are presented with standard deviations and derived from six hybridization experiments for NBK ± FBS and from two hybridization experiments for SV pgC2a and SQCC/Y1. Statistical analysis, in addition to the one provided by GCOS, was performed for NBK ± FBS by unpaired two-tailed t-test (*, p < 0.05; **, p < 0.01; ***, p < 0.001). All differentially transcribed CMEs were furthermore verified by Statistical Analysis of Microarray (SAM) [224].

Thus, the results suggest that altered transcription of at least three CME genes, i.e. ALDH4A1, HPGD, and AKR1A1 is altered at the onset of TSD. Notably, these three
changes can tentatively be ascribed to signal transduction pathways with established or plausible function in keratinocyte differentiation. Transcription of p53-transduced **ALDH4A1** is lost in serum-differentiated NBK as well as in both transformed cell lines which might reflect p53-status in all three cases: p53 is inactivated in SVpgC2a, absent in SqCC/Y1 and p53 effects appear to be counteracted by p63 during keratinocyte differentiation [6, 77, 81, 225, 226]. HPGD is transduced by TGF-β, a TSD-inducing serum component, and considered to fulfill a tumor suppressor function in many cancer types [118, 227-229]. Finally, **AKR1A1** gene expression is among others regulated by CHOP (C/EBP homologous protein) which has been shown to be upregulated during early stages of TSD in keratinocytes [230, 231]. To date, no xenobiotic activity has been reported for **ALDH4A1**, a mitochondrial enzyme catalyzing the NAD⁺-dependent second step in the degradation of proline to glutamate [232]. In contrast, HPGD is a COX-2 antagonist which, in addition to its function in the inactivation of prostaglandins, has been suggested to play a role in xenobiotic metabolism [114, 119]. Finally, **AKR1A1** codes for a ubiquitous aldehyde reductase which displays activity for both biogenic and exogenous aldehydes as well as anticancer drugs and quinones [131].

5.2.4 CME-mediated xenobiotic activity is increased at the onset of TSD (Paper III)

Taking advantage of the promiscuous substrates menadione and 9,10-PQ in combination with different relevant cofactors and inhibitors, efforts were made to assess CME-mediated xenobiotic activity in response to serum. NBK, but none of the transformed cell lines, exhibited increased menadione reduction activity in response to serum. The activity increase was characterized by dual NADPH/NADH specificity as well as high dicoumarol sensitivity, notably both properties of NQO1 [84]. Hence, the data is suggestive of NQO1 as the responsible enzyme.

NADPH-dependent 9,10-PQ reduction activity was increased in NBK and SqCC/Y1 in response to serum, but not in SVpgC2a. The activity increase was rutin-sensitive for NBK, but dicoumarol-sensitive for SqCC/Y1 which implies that the enzyme responsible for the activity increase in SqCC/Y1 is not the same as the one in NBK. The sensitivity towards rutin suggests increased AKR and/or CBR activity in serum-differentiated NBK [132, 135, 233, 234]. Considering the comparatively low activity of **AKR1A1** towards 9,10-PQ, it is unlikely that **AKR1A1** alone can account for the rutin-inhibitable fraction observed in NBK, although the observed increase in **AKR1A1**
transcription might very well translate to the activity increase observed in lysate from serum-exposed NBK (cf. Fig. 18) [131]. Apart from serum-exposed SqCC/Y1, dicoumarol did not have any effect on NADPH-dependent 9,10-PQ reduction activity. This implies that CRYZ does not contribute significantly to ortho-quinone metabolism in neither NBK nor SVpgC2a, and, with CRYZ transcripts being close to detection limit in SqCC/Y1, neither in SqCC/Y1 [101]. Finally, NADH-dependent 9,10-PQ reduction was less variable and, surprisingly, much more efficient in all cell lysates, resulting in two- to threefold higher activities. As no activity with prostaglandin F2α was detected, the NAD⁺-dependent HPGD was unlikely to be responsible for this high activity [114].

Taken together, the results on quinone reduction activity suggest considerable capacity for CME-mediated xenobiotic metabolism in buccal epithelial cells which is significantly altered in the serum-differentiated state. Notably, to attribute these effects to specific CMEs, more directed approaches are needed including e.g. immuno-depletion or siRNA techniques.

5.3 THE DUAL FUNCTION OF ADH3 AS GSNO REDUCTASE AND FORMALDEHYDE DEHYDROGENASE

Table 3: A comparison of thus far reported steady-state kinetic parameters for GSNO reduction and HMGSH oxidation by human ADH3. \(k_{\text{cat}}\) values are calculated per ADH3 monomer.

<table>
<thead>
<tr>
<th>K_m [µM]</th>
<th>k_cat [min⁻¹]</th>
<th>k_cat/K_m [min⁻¹µM⁻¹]</th>
<th>K_m [µM]</th>
<th>k_cat [min⁻¹]</th>
<th>k_cat/K_m [min⁻¹µM⁻¹]</th>
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<td>27</td>
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<tr>
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<td>90</td>
<td>4.8</td>
<td>1,760</td>
<td>370</td>
<td>[235]</td>
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<tr>
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<td>160</td>
<td>115</td>
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Initially, steady-state kinetics for HMGSH oxidation and GSNO reduction was assessed by fluorescence spectroscopy, for better sensitivity as compared to the more
commonly used absorption spectroscopy. In this study, the $K_m$ found for HMGSH was five- to tenfold lower than in previous studies, while the $k_{cat}$ as well as the kinetic parameters for GSNO reduction were mostly consistent with previously reported values (Table 3).

5.3.1 GSNO promotes ADH3-mediated oxidative reactions and is concurrently reduced under cofactor recycling (Paper IV)

ADH3-mediated alcohol (octanol, ethanol and HMGSH) oxidation was monitored in absence and presence of GSNO by fluorescence and absorption spectroscopy, taking advantage of the fact that NADH and GSNO both absorb at 340 nm while only NADH fluoresces. With GSNO being a substrate for the reverse reaction, a product inhibition pattern would have been expected. However, in presence of GSNO an initial decrease in absorbance was observed which was attributed to the reduction of GSNO, as only NAD$^+$ had been added to the reaction mixture. Fluorescence spectroscopy showed that the initial negative slope coincided with absence of net NADH formation, indicating that NADH was immediately recycled by GSNO reduction (Fig. 19). Reaction velocities could thus be calculated in absence and presence of GSNO using the molar extinction coefficients of NADH (6220 M$^{-1}$cm$^{-1}$) and GSNO (840 M$^{-1}$cm$^{-1}$), respectively.

![Fig. 19: Measurement principle of ADH3-catalyzed octanol oxidation in presence and absence of GSNO. Solid lines show reactions monitored by following absorbance at 340 nm. Dashed lines show reactions monitored by the NADH-specific fluorescence emission at 455 nm with 340 nm as excitation wavelength. In the presence of GSNO, the initial negative slope coincides with absence of net NADH production. Reactions were carried out in 0.1 M potassium phosphate buffer pH 7.5, 2.4 mM NAD$^+$, 2.0 mM octanol and 62.5 µg/ml ADH3 in absence and presence of 250 µM GSNO.](image-url)
A comparison revealed that not only was GSNO reduction facilitated by ADH3-mediated alcohol oxidation providing the reduced cofactor, but the overall reaction rates were increased, notably up to eight-fold for the substrate pair HMGSH/GSNO. The results are consistent with immediate enzyme-bound cofactor recycling, as cofactor release is a step that is partially rate-limiting in HMGSH oxidation [235]. Strikingly, this effect was more pronounced when HMGSH oxidation was assessed with crude murine liver and lung lysates instead of purified ADH3. Here, presence of GSNO increased reaction rates about 20-fold. At the same time, GSNO degradation was very low in all controls without formaldehyde, excluding a significant contribution of non-enzymatic GSNO degradation to overall GSNO decay. Hence, circumvention of cofactor release apparently gains importance in a more complex environment including other NAD+/NADH-scavenging enzymes. Notably, in presence of 1 mM ethanol instead of formaldehyde as substrate in these reactions, GSNO was still reduced, but no reaction rate increase was observed. At such low concentrations the contribution of ADH3 to ethanol oxidation is negligible, as ADH1 is the main ethanol-oxidizing enzyme in liver. Thus, in this case NADH could not be provided in ADH3-bound form and instead, the rate of GSNO reduction was limited by the rate of free NADH production following ADH1-mediated ethanol oxidation. Hence, through the proposed mechanism, enzyme-bound cofactor recycling, formaldehyde might induce rapid GSNO depletion under physiological conditions, including the typically high free NAD+/NADH ratio (Fig. 20) [182]. It appears that any source of NADH can trigger GSNO reduction but that GSNO depletion is much more rapid, when the redox cycle is constrained to ADH3.

Fig. 20: Model for formaldehyde (HCHO)-induced GSNO depletion. The spontaneously formed adduct between HCHO and GSH, HMGSH, is oxidized by ADH3 under concomitant conversion of NAD+ to NADH. The latter does not dissociate from the enzyme, but is directly used for GSNO reduction to the intermediate semimercaptal S-hydroxylaminoglutathione (GSNHOH).
5.3.2 Formaldehyde-triggered GSNO depletion, mediated by ADH3, might underlie the asthma-exacerbating effects of formaldehyde

It is highly striking that two reverse substrates of ADH3 that trigger or promote each others’ conversion have opposite effects in asthma. Formaldehyde is known to act as a bronchoconstrictor and exacerbates asthma symptoms, although the molecular causes are not well understood [171-173]. In contrast, GSNO acts as an endogenous bronchodilator in airway lining fluid, protects from hyperresponsivity, and is depleted from airways of asthmatic patients [32, 197]. Evidence for an adverse role of ADH3 in asthma was provided by a study using Adh3-deficient mice which showed that genetic ablation of ADH3 results in higher levels of SNOs in lung homogenates and associates with protection from airway hyperresponsivity [32]. Moreover, two single nucleotide polymorphisms in the ADH3 gene have been coupled to asthma susceptibility [161]. A causal relationship between formaldehyde and decreased SNO levels in the regulation of airway responsivity, with ADH3 as the interconnecting factor, has been considered, but the findings presented above provide for the first time experimental evidence for one [237]. Accordingly, under asthmatic conditions, including lung epithelial cell damage, ADH3, GSH and NAD⁺ are likely to be present in the airway lining fluid and thus, inhalation of formaldehyde might lead to rapid depletion of GSNO, resulting in bronchoconstriction and enhanced airway hyperresponsivity. In support of this hypothesis, it was shown in paper IV that exposure of cultured cells (SqCC/Y1) to formaldehyde results in a trend towards decreased SNO levels. Taken together, the presented results of paper IV are highly suggestive of an ADH3-driven mechanism underlying the well-established asthma-exacerbating properties of formaldehyde, inhalation of which would entail depletion of the protective bronchodilator GSNO. Nevertheless, the relevant experiments to prove that such a mechanism functions in vivo remain to be performed.

5.3.3 GSNO reduction results in the GSH-controlled formation of glutathione transferase (GST) inhibitors (Paper IV)

Product formation following GSNO reduction was assessed as a function of GSH concentrations. To this end, reaction mixtures were resolved on a strong anion exchange column after different reaction times, followed by (semi-)quantification by peak integration and qualitative reactant/product identification by ESI MS. The results showed equimolar consumption of NADH relative to GSNO, increasing hydroxylamine, decreasing glutathione sulfamidate formation in response to increasing
GSH concentrations, as well as glutathione disulfide as major reaction product at the highest GSH concentration. These findings support the results by Jensen et al. [28].

Fig. 21. Model for GSH-responsive product formation of ADH3-mediated GSNO reduction. In absence of GSH, ADH3-mediated GSNO reduction is fast and the intermediate S-hydroxylaminoglutathione can be spontaneously rearranged to the sulfinamide. Glutathione sulfinamide is partly hydrolyzed to the sulfinic acid which is likely to be oxidized to glutathionesulfonic acid under oxidative stress (ROS, reactive oxygen species). These three product species, glutathione sulfinamide, sulfinic acid and sulfonic acid, increasingly inhibit MGST1. In contrast, GSH at millimolar concentrations decreases the rate of ADH3-mediated GSNO reduction and the intermediate S-hydroxylaminoglutathione is intercepted by GSH to yield GSSG and NH₂OH. Invariably, NADH for GSNO reduction can be provided by oxidative ADH3 pathways, e.g. by oxidation of the glutathione adduct of formaldehyde, HMGSH.

While GSSG can be recycled to GSH by glutathione reductase and other enzymes, it was unclear whether glutathione sulfinamide, if produced under physiological conditions, would be metabolized further. Notably, in reactions with crude liver lysate instead of purified ADH3 a similar product formation pattern in response to GSH concentrations was observed, indicating that glutathione sulfinamide is a metabolic dead-end. The only product formed from glutathione sulfinamide that could be detected, generally after longer reaction times and at low GSH/GSNO ratios, was its spontaneous hydrolysis product glutathione sulfonic acid. As glutathionesulfonic acid is a well-known inhibitor for GSTs, it was assessed whether glutathione sulfinamide and
glutathione sulfinic acid equally inhibited MGST1 [143, 144]. Both compounds were found to inhibit MGST1 with IC\textsubscript{50} values of 48 µM (sulfinamide) and 22 µM (sulfinic acid), close to the IC\textsubscript{50} value of the established inhibitor glutathionesulfonic acid (5.3 µM) and considerably stronger than GSNO with an IC\textsubscript{50} of 520 µM.

Taken together, the results imply that, under physiological conditions, GSNO reduction is decelerated by intracellular GSH (5-10 mM) which favors the interception of the intermediate semimercaptal by GSH and leads to the formation of GSSG as major product. However, severe oxidative stress, GSH depletion under disease conditions, or varying intracellular distribution of GSH might lead to the formation of the GST inhibitors glutathione sulfinamide and sulfinic acid and thus exacerbate toxic responses (Fig. 21) [238, 239].

5.3.4 Different substrate analogues as inhibitors of ADH3-mediated GSNO reduction (Paper V)

The above presented evidence suggests that ADH3 exhibits a Janus face. While ADH3 is known as a central player in the cellular protection against formaldehyde, it has now emerged that ADH3 might bring about adverse effects through its function as GSNO reductase, particularly evident in asthma [32]. First, ADH3 can lead to irreversible GSNO depletion if provided the appropriate cofactor NADH. Second, GSNO reduction can lead to the formation of GST inhibitors. In the light of these adverse effects, a specific ADH3 inhibitor might be of direct clinical use, particularly for the treatment of asthma, but possibly also for other disease conditions. Thus, a range of substrate analogues, including medium-chain fatty acids and GSH-derived compounds, was tested as inhibitors of ADH3-mediated GSNO reduction. Notably, of all known ADH3-substrates, the GSH-derived substrates HMGSH and GSNO display the highest affinity, as reflected by (sub-)micromolar K\textsubscript{m}-values reported in paper IV and previous studies (cf. Table 3). Medium-chain ω-hydroxy fatty acids, particularly 10-hydroxydecanoic acid and 12-hydroxydodecanoic acid exhibit higher, but still micromolar, K\textsubscript{m} values (paper V) [158, 240]. Hence, analogues for both substrate types were tested as inhibitors.

Irrespective of substrate analogue tested, with GSNO as variable substrate and a constant concentration of NADH, in most cases the model for non-competitive inhibition yielded the best data fit. In contrast, dodecanoic acid has previously been shown to competitively inhibit ADH3-mediated oxidative reactions with respect to
HMGSH and 12-hydroxydodecanoic acid reflecting that dodecanoic acid binds into the active site, as also evident from the crystal structure [166, 167, 235]. Notably, for alcohol dehydrogenase-catalyzed reactions, it is not uncommon for substrate analogues to show competitive inhibition in one reaction direction but uncompetitive or non-competitive inhibition in the reverse direction. This observation is frequently explained by the inhibitor’s preference for binding one of the two possible enzyme-cofactor complexes (the enzyme-NAD$^+$ or the enzyme-NADH complex) [167, 241]. For the substrate analogues tested here, this suggests that the formation of enzyme-NAD$^+$-inhibitor complexes is favored over enzyme-NADH-inhibitor complexes.

Table 4. Medium-chain fatty acids as inhibitors for ADH3-mediated GSNO reduction.

<table>
<thead>
<tr>
<th>fatty acid</th>
<th>$K_i$ [µM]$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>octanoic (caprylic) acid</td>
<td>909 ± 69</td>
</tr>
<tr>
<td>nonanoic (pelargonic) acid</td>
<td>621 ± 63</td>
</tr>
<tr>
<td>decanoic (capric) acid</td>
<td>231 ± 11</td>
</tr>
<tr>
<td>undecanoic acid</td>
<td>190 ± 22</td>
</tr>
<tr>
<td>dodecanoic (lauric) acid</td>
<td>177 ± 26</td>
</tr>
<tr>
<td>tridecanoic acid</td>
<td>224 ± 6</td>
</tr>
<tr>
<td>decanedioic (sebacic) acid</td>
<td>437 ± 77</td>
</tr>
<tr>
<td>dodecanedioic acid</td>
<td>173 ± 18</td>
</tr>
<tr>
<td>2-aminodecanoic acid</td>
<td>NI$^b$</td>
</tr>
</tbody>
</table>

$^a$ evaluated with the model for non-competitive inhibition

$^b$ no inhibition observed with 400 µM

The inhibition constants for fatty acids were in the micromolar range, higher than but close to the $K_m$-values of the corresponding substrates, and showed a clear dependency on chain length. The most efficient inhibitors were found to be undecanoic acid, dodecanoic acid and dodecanedioic acid, with no significant differences in inhibition constant (Table 4). Evident from the ADH3 structure with 12-hydroxydodecanoic acid, the binding of $\omega$-hydroxy fatty acids relies on interactions of the carboxyl group with the anion binding pocket (Q111, R114, and K283 from the other subunit) at the base of the active site, interactions of the long aliphatic chain with
hydrophobic residues lining the active site towards the top, and finally the active site zinc [166]. Non-substituted fatty acids cannot interact favorably with the active site zinc as they are devoid of the nucleophilic group on the $\omega$-carbon. Hence, in efforts to increase affinity by adding a zinc binding moiety on the $\omega$-carbon of the inhibitor, two maximally oxidized substrate analogues, decanedioic acid and dodecanedioic acid, were tested as inhibitors. However, this did not improve affinity and actually had an unfavorable effect in the case of decanedioic acid. Furthermore, the introduction of an amino group in decanoic acid (2-aminodecanoic acid) abolished inhibition. Taken together, these results suggest that the active site, when binding fatty acids, can accommodate a second polar group at the $\omega$-carbon if the chain length is optimized for maximal hydrophobic interactions, but overall binding affinity is thereby not improved.

Table 5. GSH derivatives as inhibitors for ADH3-mediated GSNO reduction.

<table>
<thead>
<tr>
<th>GSH (derivative)</th>
<th>$K_i$ [mM]$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>21 ±3</td>
</tr>
<tr>
<td>S-methylglutathione</td>
<td>15 ±1</td>
</tr>
<tr>
<td>S-acetamidoglutathione</td>
<td>6.1 ± 0.6</td>
</tr>
<tr>
<td>GSSG</td>
<td>&gt; 50$^b$</td>
</tr>
<tr>
<td>glutathione sulfonic acid</td>
<td>NI$^c$</td>
</tr>
</tbody>
</table>

$^a$ evaluated with the model for non-competitive inhibition
$^b$ weak inhibition with 10 and 20 mM
$^c$ no inhibition observed with 10 mM

In contrast to the Michaelis constants for the high-affinity substrates HMGSH and GSNO, the $K_i$ for GSH is in the millimolar range (papers IV and V, Table 5) [28]. The inhibitory effect was only marginally improved by substitution of the sulphydryl proton with a methyl group. Hence, removal of the binding group to the active site zinc reduces affinity by three orders of magnitude for GSH-derived ligands. The addition of a potential zinc binding moiety to the glutathione scaffold was attempted by introducing an acetamido group, where either the carboxyl oxygen or the amino nitrogen is in theoretical zinc coordination distance. This improved inhibitory capacity threefold in comparison to GSH, but $K_i$ was still in the millimolar range, possibly due to unfavorable sterical or electrostatic interactions emanating from the electronegative...
atom that had remained uncoordinated to the zinc. Finally, very weak or no inhibition was observed with glutathione disulfide and glutathionesulfonic acid (Table 5).

Protein-ligand docking simulations for ADH3 using GSNO and S-methylglutathione (MGSH) as ligands were performed to address the considerable discrepancies in affinity. The results suggested that GSNO binds in a comparably well-ordered fashion in a manner similar to HM GSH. In contrast, the binding of MGSH was considerably less ordered and three independent docking simulations resulted in distinctly different ligand conformations including one where the ligand had escaped the active site. The difference in calculated binding free energies from the lowest-energy conformations of each docking simulation translated to by two magnitudes decreased dissociation constants which is qualitatively consistent with the experimental $K_m$ and $K_i$ values. Taken together, the findings imply that, for GSH-derived ADH3 ligands, a nucleophilic group in coordination distance of the active site zinc is imperative for efficient binding. This is in contrast to the binding of medium-chain fatty acid derivatives where the active site zinc plays an inferior role in binding and the addition of zinc-binding moieties does not improve overall binding affinity.
Assessment of differential gene expression in SVpgC2a and SqCC/Y1 relative to NBK by 2D-PAGE and DNA microarray analysis, assisted by selected bioinformatics tools, lead to the identification of multiple previously proposed and potential novel markers for BSCC. The markers included such at transcript and protein level as well as novel integrative markers as GO categories or aberrant molecular networks with their central regulator genes, representing potential tumor suppressor/promoter genes. A significant part of the single markers had previously been implied for buccal or oral carcinogenesis, supporting the overall approach. Hence, the culture of NBK, SVpgC2a and SqCC/Y1 provides a good model system for buccal carcinogenesis mechanisms, including for the testing of -omics data mining as novel diagnostic strategy. The methodology put forward contemplates the concept of multiple markers or aberrant molecular networks as more sensitive diagnostic and prognostic tools than single markers. Furthermore, the approach appears useful under circumstances of limited material, e.g. for individualized therapy.

Post-confluent culture in absence and presence of FBS was successfully used to model different strata of the buccal epithelium, as indicated by the CFE assay, changes in cell morphology and altered expression of established markers of proliferation and TSD. Notably, the overall results suggest that more than one growth protocol is required to mimic the complete buccal epithelium in vitro. Post-confluent culture of NBK for three days in presence of 5% FBS appears to effectively capture the onset of TSD. As to CME transcription in association with proliferation and TSD, the results of paper II argued for a positive correlation of ADH3 transcription with proliferation as opposed to a negative correlation with the process of TSD. In addition, collective assessment of CME gene expression indicated that transcription of three more CME genes, namely ALDH4A1, AKR1A1 and HPGD, is altered at the onset of TSD. Enzymatic quinone reduction activities in crude cell lysates suggested considerable capacity for CME-mediated xenobiotic metabolism in NBK as well as increased NQO1 and AKR activity in post-confluent serum-differentiated NBK. Although the results need to be verified by e.g. immunodepletion or siRNA techniques, the presented work identified AKR1A1 and NQO1 as potential targets for neutralizable anti-neoplastic drugs in the treatment of BSCC.
The high cytosolic free NAD$^+/\text{NADH}$ ratio does not favor reductive reactions that are dependent on NADH, and NADPH cannot efficiently be used by ADH3 [29, 182]. The results of paper IV showed that, in vitro, an unfavorable NAD$^+/\text{NADH}$ ratio can be overcome in the presence of a substrate for an ADH3-mediated oxidative reaction by immediate enzyme-bound cofactor recycling. This was most efficiently achieved by formaldehyde in the form of its GSH adduct HMGSH, but in principle, this could be triggered by any source of NADH. Furthermore, formaldehyde exposure of SqCC/Y1 led to a significant decrease in total cellular SNO content. Taken together, the results provided indirect evidence for formaldehyde as a physiological trigger of GSNO depletion. The mechanism put forward offers a molecular explanation for the asthma-exacerbating effects of formaldehyde, where inhalation of formaldehyde leads to depletion of GSNO, an endogenous bronchodilator, in airway lining fluid.

Moreover, ADH3-mediated GSNO reduction can lead to the formation of glutathione sulfamidam and sulfenic acid, both potent inhibitors of GST activity. However, typical cellular GSH concentrations in the millimolar range decelerated the ADH3-catalyzed reaction and precluded the rearrangement to the sulfanamide by quantitative interception of the intermediate semimercaptale and formation of GSSG. Nevertheless, under conditions of low local GSH concentrations, e.g. following formaldehyde scavenging or under severe oxidative stress, toxic responses might be aggravated by the formation of GST inhibitors. The overall results imply that ADH3 exhibits a Janus face, as the major formaldehyde scavenger on the one hand, but with adverse effects due to its GSNO reductase activity on the other hand.

In the light of ADH3-mediated GSNO reduction as an adverse effect, various analogues of ADH3 substrates were tested as inhibitors for ADH3-mediated GSNO reduction. Among all tested inhibitors, the most efficient were undecanoic, dodecanoic acid and dodecanedioic acid. All GSH-derived inhibitors displayed inhibition constants in the millimolar range, at least three orders of magnitudes higher than the $K_m$ values of the high-affinity substrates GSNO and HMGSH. The experimental results as well as docking simulations with GSNO and MGS suggest that for ADH3 ligands with a glutathione scaffold a zinc-binding moiety is imperative for correct orientation and stabilization of the hydrophilic glutathione scaffold within a predominantly hydrophobic active site. The results provide guidelines for future design of a specific ADH3 inhibitor with potential direct clinical use for the prevention of asthma-exacerbating effects, and possibly for various other disease conditions.
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