Identification of cytotoxic, glutathione-reactive moieties inducing accumulation of reactive oxygen species via glutathione depletion

Julian Wilkea,b, Tatsuro Kawamuraa,c, Nobumoto Watanabec,d, Hiroyuki Osadac,e, Slava Zieglera, Herbert Waldmanna,b,*

a Max Planck Institute of Molecular Physiology, Otto-Hahn-Str. 11, 44227 Dortmund, Germany
b Technical University of Dortmund, Emil-Figge-Str. 72, 44221 Dortmund, Germany
c RIKEN-Max Planck Joint Research Division for Systems Chemical Biology, RIKEN Center for Sustainable Resource Science, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
d Bio-Active Compounds Discovery Research Unit, RIKEN Center for Sustainable Resource Science, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
e Chemical Biology Research Group, RIKEN Center for Sustainable Resource Science, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

Article info
Article history:
Received 31 July 2017
Revised 25 October 2017
Accepted 3 November 2017
Available online 4 November 2017

Keywords:
Glutathione depletion
Reactive oxygen species
Small molecules
Cytotoxicity

Abstract
Reactive oxygen species (ROS) play an essential role in the survival and progression of cancer. Moderate oxidative stress drives proliferation, whereas high levels of ROS induce cytotoxicity. Compared to cancer cells, healthy cells often exhibit lower levels of oxidative stress. Elevation of cellular ROS levels by small molecules could therefore induce cancer-specific cytotoxicity. We have employed high-throughput phenotypic screening to identify inducers of ROS accumulation. We found 4,5-dihalo-2-methylpyridazin-3-one (DHMP) and 2,3,4,5(6)-tetrachloro-6(5)-methylpyridine (TCMP) moieties to strongly deplete GSH, to cause ROS accumulation and to induce cell death. Small molecules containing these fragments most likely share the same properties and should therefore be carefully considered in the development of bioactive molecules.

1. Introduction
Reactive oxygen species (ROS), i.e. radical and non-radical reactive molecules comprising oxygen, such as superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radicals (OH), are by-products of the cellular metabolism, largely originating from the mitochondria, and play a role as signaling molecules in a variety of cellular processes.¹,² However, at high levels, ROS can induce cytotoxicity, as they can directly react with biomolecules, thereby causing e.g. DNA mutations, oxidation of proteins and lipid peroxidation.³ In many cancer cell lines the redox balance is shifted towards higher ROS levels when compared to non-malignant cells.⁴,⁵ Moderately elevated ROS levels promote cancer survival by increasing DNA mutation rates. However, further elevation has detrimental impact on cells as it can cause cell death.⁶ Therefore, many cancer cells constantly exploit a large portion of their antioxidative response measures, which renders them more sensitive towards further increase in ROS concentrations.⁷,⁸ Thus, ROS-inducing small molecules may induce cancer-selective cell death.¹ We identified compounds increasing cellular ROS levels by means of a phenotypic screening.

Strong depletion of cellular GSH was observed after treatment with hit compounds containing either a 4,5-dihalo-2-methylpyridazin-3-one (DHMP) or 2,3,4,5(6)-tetrachloro-6(5)-methylpyridine (TCMP) moiety. Herein we show that DHMP and TCMP moieties are reactive with GSH in solution and deplete GSH in cells, thereby inducing ROS accumulation, which might trigger the observed cytotoxicity.

2. Results
To identify small-molecule inducers of ROS in cells, a phenotypic assay, adapted from Adams et al., in U-2OS cells using the ROS indicator CM-H₂DCFDA was employed to screen a library of 186,117 compounds.⁹ As GSH is the most abundant antioxidant and GSH depletion by small molecules is directly linked to ROS accumulation, hit compounds were then tested for depletion of cellular GSH.¹⁰–¹² Several compounds depleted GSH in U-2OS cells (Fig. S1) and more than half of the 30 most active GSH depleters bore a 4,5-dihalo-2-methylpyridazin-3-one (DHMP) moiety, while 4 compounds contained a 2,3,4,5(6)-tetracloro-6(5)-methylpyridine (TCMP) moiety (Table S1). Additionally, another 7 compounds bore a 5-chloro-2-(methylsulfonyl)pyrimidin-4-ylmethanone moiety,
however, this fragment was already described in the context of cellular GSH depletion.\textsuperscript{13} 4,5-dichloro-2-methylpyridazin-3-ones and 2,3,4,5-tetrachloropyridines have recently been described as cytotoxic fragments with an unknown mode of action.\textsuperscript{14} To explore the link between DHMP and TCMP moieties, ROS accumulation, GSH depletion and cytotoxicity, six representative compounds with different ROS-inducing activities were selected for in-depth characterization (Fig. 1).

Accumulation of ROS after one hour of incubation with the six test compounds was also explored in HeLa cells. The GSH depleter 1-chloro-2,4-dinitrobenzene (CDNB), which induces ROS accumulation via depletion of cellular GSH, was included as positive control (Fig. 2a). Strong ROS induction was observed at 10 \textmu M for DHMP01 (63.96\% ± 18.47), DHMP02 (78.23\% ± 8.31), TCMP01 (73.78\% ± 13.35) and TCMP02 (98.33\% ± 15.68), whereas DHMP03 (9.73\% ± 7.21) and TCMP03 (12.14\% ± 14.83) did not affect cellular ROS levels (Fig. 2b–d).

Next, the cytotoxic effect of the test compounds was examined by means of real-time live-cell imaging via propidium iodide staining to detect loss of cell membrane integrity. 5 \textmu M of DHMP01 or TCMP01 and 10 \textmu M of DHMP02 or TCMP02 were cytotoxic in HeLa cells (Fig. 3a–b and d–e). Cells treated with 10 \textmu M of DHMP01, DHMP02 or TCMP01 showed severe changes in cellular morphology already after 2 h incubation, indicating a rapid cytotoxic effect (Fig. S2a). DHMP03 and TCMP03 did not influence cellular viability (Figs. 3c,f and S2). The induction of ROS accumulation and cytotoxicity by the test compounds strongly suggests a causative link between oxidative stress and cell death.

To address this assumption, HeLa cells were incubated with 5 mM of the antioxidant N-acetylcysteine (NAC) prior to the addition of DHMP01 or TCMP01 at cytotoxic concentrations (Fig. 4a and Fig. S3). Preincubation with NAC rescued the cytotoxic influence of both compounds along with the absence of morphological changes that were observed after treatment (Fig. 4b). Additionally, the impact of a co-treatment of the compounds with the GSH synthesis inhibitor \(L\)-buthionine sulfoximine (BSO) was analyzed. Treatment of HeLa cells with moderately cytotoxic concentrations of the test compounds and subcytotoxic concentrations of BSO enhanced the cytotoxicity synergistically (Fig. 4c and d). Taken together, the cytotoxicity rescue by antioxidants and the synergy with a GSH synthesis inhibitor indicate a possible link between redox modulation and cell death induced by DHMP01 and TCMP01.

Consequently, the influence on the total cellular GSH content was determined after 1 h of incubation with 10 \textmu M of each test compound in HeLa cells. Both non-cytotoxic and ROS-inactive compounds DHMP03 and TCMP03 did not influence cellular GSH levels. DHMP01, DHMP02 and TCMP02 decreased the total GSH content approximately by half, similarly to the known GSH depleting compound CDNB (Fig. S4). TCMP01 did not show any GSH depletion, although both ROS induction and cytotoxicity were observed at the applied concentration of 10 \textmu M.

The depletion of GSH by the test compounds may depend on the presence of certain enzymes, either catalyzing the reaction with GSH or transforming the test compounds into reactive metabolites. To assess the reactivity towards GSH directly, all test compounds were incubated in solution with 5 mM GSH, approximately

![Fig. 1. Chemical structures of (a) 4,5-dihalo-2-methylpyridazin-3-ones (DHMPs) and (b) 2,3,4,5(6)-tetrachloro-6(5)-methylpyridines (TCMPs) and their representative test compounds (c and d, respectively).](image-url)
دریافت فوری متن کامل مقاله
امکان دانلود نسخه تمام متن مقالات انگلیسی
امکان دانلود نسخه ترجمه شده مقالات
پذیرش سفارش ترجمه تخصصی
امکان جستجو در آرشیو جامعی از صدها موضوع و هزاران مقاله
امکان دانلود رایگان ۲ صفحه اول هر مقاله
امکان پرداخت اینترنتی با کلیه کارت های عضو شتاب
دانلود فوری مقاله پس از پرداخت آنلاین
پشتیبانی کامل خرید با بهره مندی از سیستم هوشمند رهگیری سفارشات