Heterocyclic cyclohexanone monocarbonyl analogs of curcumin can inhibit the activity of ATP-binding cassette transporters in cancer multidrug resistance

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ABSTRACT

Curcumin (CUR) is a phytochemical that inhibits the xenobiotic ABC efflux transporters implicated in cancer multidrug resistance (MDR), such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated proteins 1 and 5 (MRP1 and MRP5). The use of CUR in the clinic however, is complicated by its instability and poor pharmacokinetic profile. Monocarbonyl analogs of CUR (MACs) are compounds without CUR's unstable β-diketone moiety and were reported to have improved stability and in vivo disposition. Whether the MACs can be used as MDR reversal agents is less clear, as the absence of a β-diketone may negatively impact transporter inhibition. In this study, we investigated 23 heterocyclic cyclohexanone MACs for inhibitory effects against P-gp, BCRP, MRP1 and MRP5. Using flow cytometry and resistance reversal assays, we found that many of these compounds inhibited the transport activity of the ABC transporters investigated, often with much greater potency than CUR. Overall the analogs were most effective at inhibiting BCRP and we identified three compounds, A12 (2,6-bis((E)-2,5-dimethoxy-benzylidene)cyclohexanone), A13 (2,6-bis((E)-4-hydroxyl-3-methoxybenzylidene)-cyclohexanone) and B11 (3,5-bis((E)-2-fluoro-4,5-dimethoxybenzylidene)-1-methylpiperidin-4-one), as the most promising BCRP inhibitors. These compounds inhibited BCRP activity in a non-cell line, non-substrate-specific manner. Their inhibition occurred by direct transporter interaction rather than modulating protein or cell surface expression. From these results, we concluded that MACs, such as the heterocyclic cyclohexanone analogs in this study, also have potential as MDR reversal agents and may be superior alternatives to the unstable parent compound, CUR.

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1. Introduction

Multidrug resistance (MDR) in cancer chemotherapy is a phenomenon wherein patients do not respond to treatment from multiple anticancer drugs of diverse structures and mechanisms of action [1,2]. It is the primary cause of chemotherapy failure and is the reason for the continued high mortality rate, especially of advanced-stage, metastatic cancers [2]. Many mechanisms have been identified that cause or contribute to cancer MDR. One of the best known and most extensively studied is the active efflux of anticancer drugs out of tumor cells by promiscuous transmembrane proteins called ATP-binding cassette (ABC) transporters [3].

ABC transporters are a large family of evolutionarily conserved proteins with 49 members present in humans [4]. A subset of this family called xenobiotic efflux pumps, have been implicated in MDR, with the best characterized being P-glycoprotein (P-gp), Breast cancer resistance protein (BCRP) and Multidrug resistance-associated protein 1 (MRP1) [5]. These transporters are expressed in pharmacological barriers (e.g., blood–brain-barrier, gut, kidneys) and appear to function mainly to limit exposure of the body or organs to potentially harmful foreign substances [5]. However, these transporters have also been shown to be expressed in tumor tissue in a number of cancers, and were found in vitro to actively efflux a wide range of clinically important anticancer drugs, including anthracyclines, kinase inhibitors and taxels [5–8]. Expression levels of these transporters in tumors have been negatively correlated with chemotherapy response, and their inhibition or gene-silencing in preclinical models of MDR resensitises cells and tumor xenografts to chemotherapy

[9–11]. Unsurprisingly, a number of attempts have been made to block the activity of these transporters in the clinic.

Since the discovery of P-gp in 1976, three generations of P-gp inhibitors have undergone clinical trials [12]. None of these compounds however, have been approved for clinical use. Aside from some shortcomings in study design, the toxicity of the inhibitors themselves contributed to the failure of these trials. For example, first-generation P-gp inhibitors (e.g., verapamil and cyclosporine A), were used at concentrations that caused toxicity from their primary pharmacology (cardiac arrhythmia and immunosuppression, respectively); while second-generation inhibitors (e.g., valspodar) interfered with the clearance of co-administered anticancer drugs by the CYP450 enzymes [12–14].

Potentially safer alternatives that have been investigated include various phytochemicals [15]. Some of these secondary plant metabolites can modulate the activity of ABC transporters and are ubiquitously present in our diet. One of the most promising phytochemicals is curcumin (CUR), found in the turmeric spice, and a common component of curry powder [16]. CUR can inhibit the activity of P-gp, BCRP and MRP1, and has been shown by our group to also inhibit MRP5, a transporter implicated in pancreatic cancer resistance to gemcitabine and 5-fluorouracil therapy [15,17]. CUR is currently labeled by the FDA as GRAS (Generally recognized as safe) and is known to have intrinsic anticancer activity, making it a potential 'dual-role' antineoplastic and MDR-reversal agent [18,19]. This may make it superior to compounds that solely inhibit ABC transporters as it might also synergise with the anticancer activity of co-administered drugs. Lastly, unlike second-generation P-gp inhibitors, CUR is not a substrate of CYP450 enzymes, and thus, would be less likely to interfere with the metabolic clearance of anticancer drugs [20].

Despite CUR's excellent safety record and promising in vitro activity, a major hurdle to its clinical use is its poor pharmacokinetic profile in vivo [21]. CUR is very poorly absorbed from the gut and is rapidly metabolized, leading to low plasma concentrations after either enteral or parenteral administration [20]. This was highlighted in Phase I clinical trials where very high oral doses of 8–12 g resulted in peak plasma concentrations in the nanomolar range [22,23]. Given that CUR inhibited ABC transporter activity at micromolar concentrations in vitro, it may be difficult to achieve sufficiently high plasma concentrations to reverse MDR in vivo [17,24–26].

Over the past few years, a number of groups have overcome the problem of CUR pharmacokinetics by synthesizing derivatives that replace the β-diketone group of the compound with a mono-carbonyl spacer [27,28]. The β-diketone moiety is considered a target for liver enzymes (e.g., aldo-keto reductases) and is also thought to underlie CUR's in vitro instability [21]. The mono-carbonyl analogs of CUR (MACs) have greatly improved in vivo disposition in animal studies, do not appear to have increased toxicity, and many have similar or superior biological activity to CUR, including anticancer, antibacterial, anti-inflammatory and antioxidant effects [21,27].

Most studies on the MACs have focused on the latter and relatively little is known of their ability to inhibit ABC transporters, apart from a few studies focused on a single transporter and limited to P-gp and MRP5 [29,30]. More data is therefore needed to clarify whether the absence of the β-diketone group affects the ability of the MACs to inhibit ABC transporters and whether the MACs may be more stable alternatives to CUR as MDR reversal agents.

In this study, we examined 23 MACs with heterocyclic cyclohexanone cores for inhibitory activity against P-gp, BCRP, MRP1 and MRP5. These CUR analogs were initially synthesized and investigated for their possible cytotoxicity against hormone-therapy resistant estrogen receptor (ER)-negative breast cancer [31–33]. Many were found to be more potent antiproliferative agents than CUR against ER-negative breast cancer cell lines, such as treatment-resistant triple-negative breast cancer (TNBC) cells like MDA-MB-231 and MDA-MB-468, which also lack the HER2 and progesterone receptors [31].

By screening for transporter inhibition, we aimed to determine if these MACs could potentially be used as MDR reversal agents, and also further clarify if the absence of a β-diketone group negatively impacts on their ability as a drug class to inhibit ABC transporters. As some of these analogs have reported potent anticancer activity, there is also the possibility that dual-role antitumor agents like CUR might be identified.

2. Materials and methods

2.1. Materials

Materials were purchased from the following sources: mitoxantrone, calcein-AM, 2,7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), rhodamine-123 (RH-123), doxorubicin, verapamil, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), Ko143, DMSO, topotecan, ATP, indomethacin, creatine phosphokinase and creatine phosphate (Sigma–Aldrich, St Louis, MO); high purity CUR ($\geq$98.5%) (Enzo Life Sciences, Farmingdale, NY); MK-571, paclitaxel and etoposide (Cayman Chem, Ann Arbor, MI); raltitrexed and methotrexate (MTX) (Selleck Chem, Houston, TX); [3H]MTX (1 mCi/mL, 1:1 ethanol/water solution) from American Radiolabeled Chemicals, St Louis, MO; wortmannin and gefitinib (LC Labs, Woburn, MA); SYBR$^{®}$ green I (10,000$\times$ stock), penicillin/streptomycin, Dulbecco's modified eagle's medium (DMEM) and 1:1 DMEM/F12 (Life Technologies, Carlsbad, CA); FBS was from Medica, Auckland, NZ.

2.2. Curcumin analogs

Twenty-three heterocyclic cyclohexanone and cyclopentanone MACs were originally synthesized by our collaborator (Hawkins) for activity against breast cancer and colon cancer cell lines, as previously reported [31,33]. These analogs have cyclic ketone cores in place of the β-diketone structure (Fig. 1A). The five ketone cores are: series A – cyclohexanone; series B – N-methylpiperidone; series C – tropinone; series D – cyclopentanone and series E – butoxycarbonyl piperidone (Fig. 1B). These cores are linked by methylidene groups to two identical aromatic rings. Thirteen different aromatic groups were synthesized. These include nitrogen heterocycles, fluorine-substituted pyridines, N-methyl-pyrrole, N-methylimidazole, N-methylindole, trimethoxyphenyl and dimethoxyphenyl substituents (Fig. 1B).

2.3. Cell lines and culture conditions

Parental human embryonic kidney-293 cells (HEK/P) and Madin–Darby canine kidney II cells (MDCKII/P) and cell lines transfected with wild-type human ABC transporters MDCKII/P-gp, MDCKII/BCRP, HEK/MRP1, HEK/MRP5 were generously provided by Prof Piet Borst (The Netherlands Cancer Institute, the Netherlands). BeWo choriocarcinoma cells were from Dr Michael Steiner (The Liggins Institute, Auckland, NZ) and were originally obtained from ATCC. HEK293 and MDCKII cells were cultured in DMEM medium and BeWo cells in DMEM/F12. Complete media contains 10% FBS and 1% penicillin/streptomycin. Cells were kept in a humidified incubator at 37 °C with 5% CO_2.