EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES 27 (2006) 524–532

available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/ejps

# Kinetic validation of the use of carboxydichlorofluorescein as a drug surrogate for MRP5-mediated transport

*Susan Pratt*, Victor Chen, William I. Perry III, James J. Starling, Anne H. Dantzig*

*Lilly Research Laboratories, Lilly Corporate Center, Indianapolis IN 46285, USA*

ARTICLE INFO

*Article history:*
Received 28 July 2005
Accepted 22 September 2005
Available online 5 December 2005

*Keywords:*
Drug resistance
Multidrug resistance protein
MRP5 (ABCC5)
Methotrexate
Pemetrexed
Carboxydichlorofluorescein

ABSTRACT

Multidrug resistance protein-5 (MRP5, ABCC5) is a member of the ATP-binding cassette transporter superfamily that effluxes a broad range of natural and xenobiotic compounds such as cyclic GMP, antiviral compounds, and cancer chemotherapeutic agents including nucleoside-based drugs, antifolate agents and platinum compounds. In cellular assays, MRP5 transfectants are less fluorescent after incubation with 5-chloromethylfluorescein diacetate (CMFDA). The present study examines the uptake of a close fluorescent analog, carboxydichlorofluorescein (CDCF), and drug substrates into inside-out membrane vesicles prepared from MRP transfected cells. MRP5-mediated uptake of CDCF was ATP-dependent and GSH-independent and possessed a $K_m$ of 12 μM and a $V_{max}$ of 56 pmol/min/mg prot. Comparison of kinetic parameters with drug substrates such as methotrexate (MTX), pemetrexed (Alimta™), and the metabolite of 5-fluorouracil, 5-fluorodeoxyuridine monophosphate (5-FdUMP) ($K_m$ values of 0.3–1.3 mM) indicated that MRP5 has a 25–100-fold higher affinity for CDCF than for these drugs and that they share a common transport binding site. In addition, the potency of MRP5 inhibitors such as probenecid, MK571, and the phosphodiesterase 5 inhibitors correlated well between the uptake of CDCF and MTX. A survey of CDCF uptake by other MRPs revealed that MRP2 (ABCC2) also demonstrated ATP-dependent uptake with a $K_m$ of 19 μM and $V_{max}$ of 95.5 pmol/min/mg prot, while MRP1 (ABCC1) and MRP4 (ABCC4) had little to no uptake. Taken together, these data indicate that CDCF is a useful fluorescent drug surrogate with which to measure ATP-dependent MRP5-mediated transport.



## 1. Introduction

Transporters play an important role in the entry and exit of drugs into and out of cells that ultimately determines the pharmacokinetics and pharmacodynamics of drugs. The ATP binding cassette (ABC) transporter superfamily contains 49 mammalian plasma membrane located, energy-driven efflux transporters. Several are drug transporters whose primary role is to efflux structurally diverse compounds, usually xenobiotic molecules, out of the cell and thereby to provide tissues a protective barrier against potentially harmful chemical agents (Kruh et al., 2001; Gottesman et al., 2002; Bodo et al., 2003). A number of these transporters efflux anti-cancer agents or their drug conjugates and play a role in drug sensitivity of tumors. P-glycoprotein (ABCB1) and multidrug resistance proteins (ABCC) are transporters that are expressed in normal and tumor tissues and are capable of influencing the efficacy of cancer chemotherapeutic treatments. P-glycoprotein was the first multidrug resistance protein to be characterized and preferentially transports cationic or neutral molecules out of the

* *Corresponding author*. Tel.: +1 317 276 7419; fax: +1 317 433 7741.
*E-mail address:* pratt_susan@lilly.com (S. Pratt).

0928-0987/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejps.2005.09.012

cell. Expression of P-glycoprotein in tumor tissue correlates with decreased responsiveness to treatment with chemotherapeutic agents, and expression in drug sensitive cells results in reduced sensitivity to a broad range of drugs. P-glycoprotein is also highly expressed in the intestine, liver, and kidney, as well as the blood–brain barrier, blood–testis barrier and placenta where the transporter can limit absorption and enhance elimination of xenobiotics (Fromm, 2004).

Multidrug resistance-associated proteins (MRP1-8, ABCC1-6, ABCC10, 11 respectively) are a related family of plasma membrane ABC efflux transporters. These family members preferentially transport negatively charged molecules that may result from conjugation or phosphorylation, or co-transport neutral compounds with anions such as GSH (Haimeur et al., 2004). Although MRP proteins transport organic anions, each has its own substrate specificity. When there is substrate overlap between transport proteins, the transporters are often distinguished by their affinities for the substrates. Most MRP proteins are able to confer resistance to otherwise drug sensitive cells, and tissue-specific expression of MRP proteins contributes to the absorption, elimination and/or barrier properties of the tissues.

MRP5 is expressed in most normal tissues, and is overexpressed in colon, lung, breast and pancreatic cancers (Kool et al., 1997; McAleer et al., 1999; Konig et al., 2005; Sandusky, 2002). When transfected into drug-sensitive cells, MRP5 confers resistance to antifolate drugs such as methotrexate (MTX) and pemetrexed (Alimta™), (Pratt et al., 2005) and to nucleoside-based drugs such as 6-mercaptopurine, 6-thioguanine, 9-(2-phosphonyl-methoxyethyl)adenine (PMEA), azidothymidine (AZT), cytosine arabinoside (AraC), 5-fluorouracil (5-FU), and gemcitabine (Wijnholds et al., 2000; Davidson et al., 2002; Wielinga et al., 2002; Pratt et al., 2005). MRP5-expressing inside-out membrane vesicles demonstrate direct transport of monophosphate metabolites of nucleoside-based drugs such as 5-FU and 6-thioguanine (Wielinga et al., 2002; Pratt et al., 2005).

5-Chloromethylfluorescein diacetate (CMFDA) is a non-fluorescent, membrane permeable compound that is hydrolyzed by esterases intracellularly to a thiol-reactive, fluorescent, negatively charged intermediate that is membrane impermeable. McAleer et al. (1999) reported reduced fluorescent labeling of MRP5-transfected cells after incubation with CMFDA and proposed that MRP5 mediated efflux of the fluorescent CMFDA hydrolysis product even though coincubation of the cells with probenecid, a non-specific inhibitor of MRPs, had no effect on cellular fluorescence. Carboxydichlorofluorescein (CDCF) is an impermeable fluorescent compound that resembles the hydrolysis product of CMFDA except the sulfhydryl reactive chloromethyl group is substituted by a carboxylic acid (Fig. 1). The present study demonstrates that MRP5 transports the fluorescent compound CDCF into inside-out MRP5-expressing membrane vesicles. Consistent with an earlier report that MRP5 confers resistance to methotrexate (MTX) and pemetrexed (Pratt et al., 2005), MRP5 also is shown to transport these two cytotoxic drugs into membrane vesicles (Wielinga et al., 2005). The transport of these two drugs and that of CDCF is characterized kinetically and compared. These studies validate the use of CDCF as a drug surrogate for the study of MRP5 mediated transport.

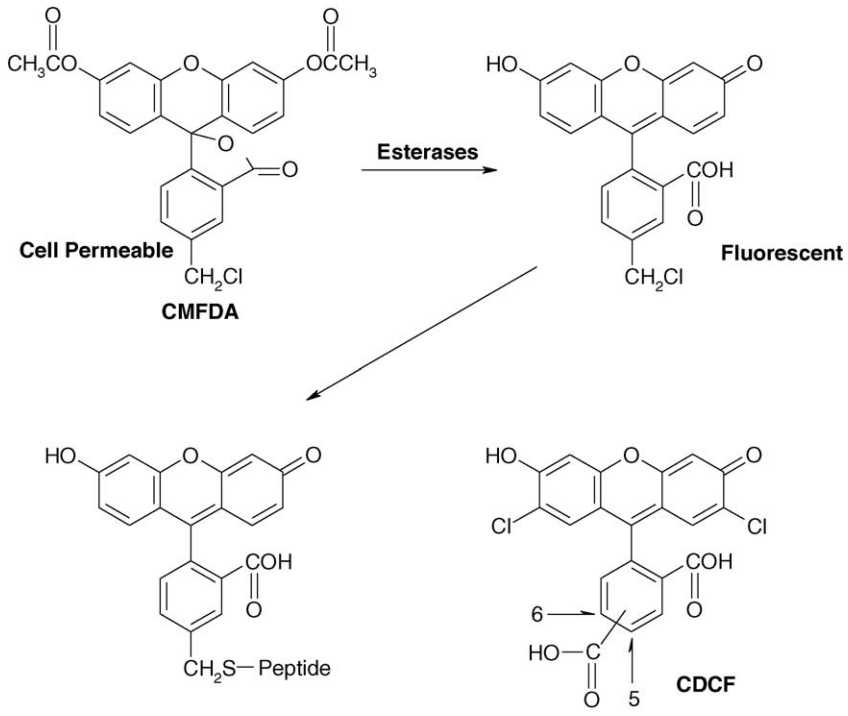


**Fig. 1 – Structures of carboxymethylfluorescein diacetate (CMFDA) and carboxydichlorofluorescein (CDCF). The non-fluorescent, membrane permeable ester CMFDA is hydrolysed to an impermeable anionic fluorescent intermediate that is reactive with thiol-groups in peptides. CDCF is a membrane impermeable, anionic fluorescent molecule structurally related to the unconjugated hydrolysis product of CMFDA.**