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Role of MRP4 and MRP5 in Biology and Chemotherapy

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ABSTRACT Nucleotide efflux (especially cyclic nucleotides) from a variety of mammalian tissues, bacteria, and lower eukaryotes has been studied for several decades. However, the molecular identity of these nucleotide efflux transporters remained elusive, despite extensive knowledge of their kinetic properties and inhibitor profiles. Identification of the subfamily of adenosine triphosphate (ATP) binding cassette transporters, multidrug resistance protein (MRP) subfamily, permitted rapid advances because some recently identified MRP family members transport modified nucleotide analogs (ie, chemotherapeutic agents). We first identified, MRP4, based on its ability to efflux antiretroviral compounds, such as azidothymidine monophosphate (AZT-MP) and 9-(2-phosphonyl methoxyethyl) adenine (PMEA), in drug-resistant and also in transfected cell lines. MRP5, a close structural homologue of MRP4 also transported PMEA. MRP4 and MRP5 confer resistance to cytotoxic thiopurine nucleotides, and we demonstrate MRP4 expression varies among acute lymphoblastic leukemias, suggesting this as a factor in response to chemotherapy with these agents. The ability of MRP4 and MRP5 to transport 3',5'-cyclic adenosine monophosphate (cAMP) and 3',5'-cyclic guanosine monophosphate (cGMP) suggests they may play a biological role in cellular signaling by these nucleotides. Finally, we propose that MRP4 may also play a role in hepatic bile acid homeostasis because loss of the main bile acid efflux transporter, sister of P-glycoprotein (SPGP) aka bile-salt export pump (BSEP), leads to a strong compensatory upregulation in MRP4 expression. Cumulatively, these studies reveal that the ATP-binding cassette (ABC) transporters MRP4 and MRP5 have a unique role in biology and in chemotherapeutic response.

KEYWORDS: Cyclic Nucleotides, Transporters, MRP4, MRP5, Physiology, Chemotherapy

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EFFLUX OF THERAPEUTIC COMPOUNDS One of the first examples of nucleoside monophosphate efflux was described for the novel nucleoside analog, tricyclic 7-deazapurine nucleoside (TCN).[1] This compound was readily transported into cells and subsequently phosphorylated by adenosine kinase to TCN-monophosphate. Once phosphorylated, intracellular TCN-monophosphate accumulated with no evidence for higher phosphorylated forms (ie, di- and triphosphate), and cytotoxicity correlated with the accumulation of TCN-monophosphate. Studies by Plagemann[1] revealed, in 4 different cell lines, that TCN-monophosphate was "released" into the medium and that the amount released into the medium varied between cell types. Release of the TCN-monophosphate into the medium showed saturable kinetics and was inhibited by depletion of cellular energy (ie, adenosine triphosphate [ATP]) and chemical inhibitors such as the organic anion transport inhibitor, probenecid. These studies suggested that the removal of monophosphorylated forms from cells was by active transport and not due to cell leakiness.[2]

Subsequent studies with the modified thymidine analog azidothymidine (AZT) revealed that AZT-monophosphate could be "secreted" from many different cell types; however, the efficiency of secretion varied among cells.[3,4] Like TCN-monophosphate, AZT-monophosphate secretion was not due to leakage or cytotoxicity and was specific for AZT-monophosphate, as higher nucleotide derivatives of AZT were not found in the extracellular medium.[4] Subsequent studies by Fridland and colleagues[5] confirmed that AZT-monophosphate was effluxed. Notably, the phenomenon of AZT-monophosphate efflux appeared inducible as longer incubations with AZT resulted in an increased rate of AZT-monophosphate efflux. Other investigators have observed a similar "induction" of drug egress.[3] Because AZT cytotoxicity is related to accumulation of AZT-monophosphate one could speculate that this active transport of drug from cells is a cytoprotective mechanism.[3-7]

Further evidence for efflux of therapeutic monophosphorylated nucleotides was obtained after the development of novel phosphonate-derived nucleoside drugs. These compounds were essentially stable monophosphorylated nucleotide analogs. One such compound, 9-(2-phosphonylmethoxyethyl) adenine (PMEA) was cytotoxic to cells[8] and resembled the natural nucleotide adenosine monophosphate. Cells with acquired resistance to PMEA cytotoxicity displayed decreased drug

accumulation.[9,10] Notably, the accumulation of PMEA in PMEA-resistant cells could be increased by the addition of indomethacin, an inhibitor of efflux.[10] These cells with decreased accumulation of PMEA exhibited increased energy-dependent egress of PMEA as well (unpublished observations[10]). Cumulatively, these studies indicated that the efflux of monophosphorylated nucleotide drugs occurs by a process that is not due to cell leakiness and suggested the existence of an active transport process.

NATURAL NUCLEOTIDE EFFLUX SYSTEMS

Shortly after Sutherland first described the biosynthesis of the intracellular "second messenger," 3',5'-cyclic adenosine monophosphate (cAMP), in response to hormonal stimulation,[11] he observed not only increased intracellular cAMP but also a concurrent rise in extracellular cAMP, suggesting extracellular cAMP played a role in cell communication. This process does occur in lower eukaryotes. For instance, in the slime mold Dictyostelium discoidium, extracellular cAMP plays a critical role in mediating the developmental transition from the amoeboid to the multicellular form because extracellular cAMP binds to cell-surface cAMP receptors, leading to chemotaxis and aggregation of single-celled organisms into a migrating slug.[12] Similarly, extracellular cAMP plays an important role in Xenopus oocyte meiosis as the application of extracellular cAMP arrests the maturation of the oocyte.[13-15] Furthermore, in other lower animals such as the frog, the intracellular level of cAMP is critical in controlling hormonal maturation of oocytes.[14-16] In mammals, cAMP is involved in a variety of responses. For example, in some cell types, elevation of intracellular cAMP leads to an inhibition of cell replication.[17] Furthermore, cAMP undergoes a tightly regulated rise and fall during the cell cycle,[18] which indicates that intracellular cAMP levels plays a role in cell cycle progression.

The efflux of cAMP in response to hormonal stimulation appears ubiquitous. For example, parathyroid hormone has been shown to stimulate both the synthesis and secretion of cAMP into the urine from the human kidney.[19] Also, glucagon stimulation of rat liver[20] and beta-adrenergic agonist stimulation of fat pads,[21] the heart,[22] and cervical ganglion lead to cAMP efflux.[23] Moreover, beta-adrenergic stimulation of mammalian reticulocytes leads to efflux of cAMP, and this occurs against a concentration gradient suggesting a process requiring expenditure of cellular energy.[24]

What is the biological role of cAMP efflux? Efflux of cAMP may be viewed as either controlling the intracellular concentration of cAMP or providing an extracellular signal (eg, adenosine) that may act in either an autocrine or paracrine fashion at specific purinergic receptors. In some model cell systems, such as cultured fibroblasts[25,26] and smooth muscle cells,[27] cAMP efflux plays a minor role in regulating the intracellular level of cAMP because the cyclic nucleotide efflux inhibitor probenecid did not increase intracellular cAMP levels compared with cells that were not treated with probenecid.[28] Thus, on the basis of these findings, it was concluded that, in these cell types, phosphodiesterases predominate over efflux in regulating the intracellular cAMP levels. However, in avian and mammalian erythrocytes, cyclic nucleotide efflux plays an important role in controlling intracellular levels.[24,29] Extracellular cAMP might also play a role in sending a signal to adjacent cells. Studies of polymorphonuclear leukocytes reveal that their adhesion to endothelial cells is controlled by extracellular cAMP. In these studies, neutralization of extracellular cAMP by an antibody raised against cAMP resulted in greater leukocyte adherence to endothelial cells. This finding implicates extracellular cAMP as a mechanism to control leukocyte adherence to endothelial cells.[30]

Although the cAMP efflux carrier reportedly effluxes other anionic substances,[31] it is possible that another efflux transporter with similar properties transports these other compounds. For instance, it is well known that 3',5'-cyclic guanosine monophosphate (cGMP) is effluxed, and this process occurs in many tissues.[32-35] Moreover, its efflux, like cAMP is also inhibited by probenecid,[33-37] a finding that suggests the cAMP and cGMP transporters are either the same or very similar molecules. Studies by Boadu and Sager[38] clearly demonstrated that cGMP efflux occurs in human erythrocyte membrane vesicles.

Cyclic GMP is an important mediator of the effects of nitric oxide (NO) and also has demonstrated effects on chloride ion transport in the kidney.[39] In the liver, large amounts of NO are produced, secondary to activation of NO synthase, after treatment with cytokines and lipopolysaccharide.[40] Hepatic NO activates soluble guanylate cyclase, leading to an elevation of intracellular cGMP and release of extracellular cGMP. The release of cGMP was inhibited by probenecid. Further studies have demonstrated that cGMP is secreted in vascular smooth muscle, endothelial cells, and fibroblasts.

How do we know that the appearance of extracellular cyclic nucleotides, either cAMP or cGMP, is due to efflux and not simply due to membrane leakage or altered membrane permeability? First, efflux of cAMP from mammalian cells requires an intracellular energy source like ATP.[41-44] Second, compounds such as the organic anion transport inhibitor probenecid[28, 41, 45, 46] and prostaglandins A1 and E1 [28, 31, 41, 42, 47-49] inhibit cAMP efflux and cGMP efflux but have no direct effect on the intracellular level of either ATP or the formation of cAMP. Moreover, prostaglandin A1 inhibition of cAMP efflux is independent of its effects on membrane fluidity.[49] Further studies have suggested that a cyclic nucleotide efflux transporter transports multiple anionic substrates. For instance, studies by Henderson and Strauss[31] demonstrated that the cAMP transporter also transports the bile acid, cholate, and this bile acid may have relevance to transporter regulation (see **Figure 1**). Moreover, the mammalian kidney, besides effluxing cAMP, can also efflux 3',5' cyclic phosphorothioates, and this efflux is also

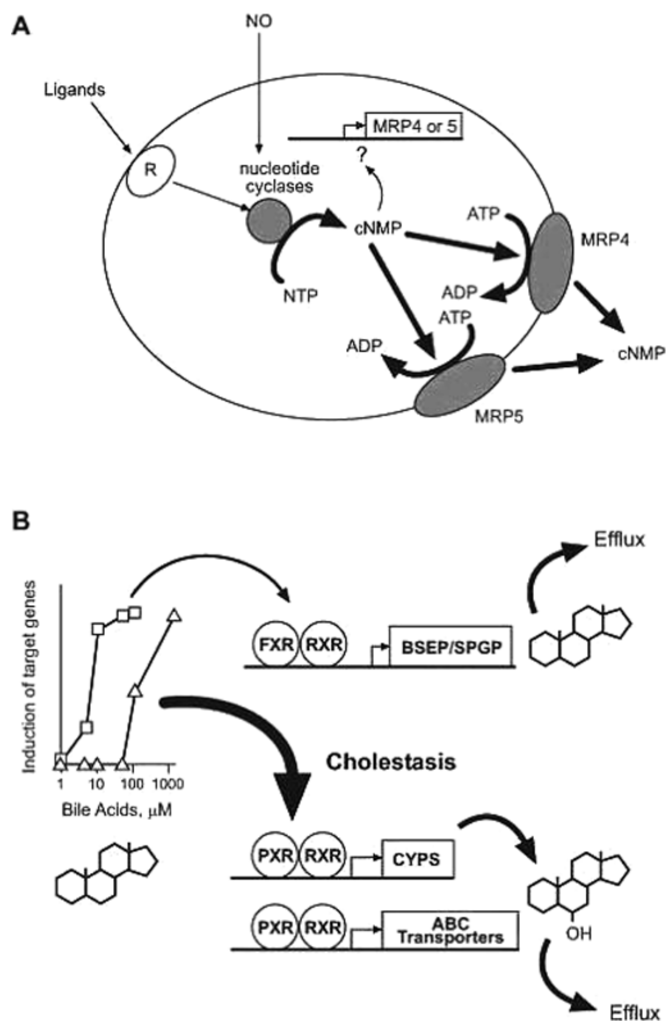

Figure 1. Function and regulation of nucleotide effluxing MRPs. MRP4 and MRP5 are shown on the membrane of the cell. (A) Extracellular signals can activate via their receptors (R) nucleotide cyclases, which lead to an elevation in cyclic nucleotide (cNMP); alternatively nitric oxide (NO) activates a similar pathway leading to the conversion nucleotide monophosphate (NMP) to cyclic nucleotide monophosphate (cNMP). The accumulated cNMP is effluxed out of the cell by either MRP4 and/or MRP5. The energy for the efflux is provided by ATP hydrolysis. We also hypothesize that cNMP may play a role in the transcriptional regulation of MRP4 and/ or 5. (B) High levels of hepatic bile acids can lead to the upregulation of MRP4. We propose that MRP4 activation occurs though bile-acid activation of the orphan nuclear receptor pregnane-X-receptor (PXR) or steroid and xenobiotic receptor (SXR).

inhibited by probenecid.[46,50] Thus, based on the requirements for cellular energy, specific chemical inhibition, and the ability to transport diverse substances, it seems likely that cyclic nucleotides are effluxed by an ABC transporter that accommodates anions.

THE ROLE OF MRP4 AND MRP5 IN NUCLEOTIDE EFFLUX

Among the large ABC transporter family, the multidrug resistance related protein (MRP) subfamily currently has 9 members (for an overview of the ABC-family members and nomenclature see http://nutrigene.4t.com/human abc.htm).[51,52] Although many are functionally uncharacterized, some have been shown to transport anionic substances against a concentration gradient with the energy for transport supplied by ATP hydrolysis.[53,54] Recent studies have demonstrated that 2 structurally similar members of the MRP family, MRP4 and MRP5, are capable of transporting therapeutic nucleoside-based compounds.[55-57] For instance, a PMEA-resistant lymphblastoid cell line was demonstrated to transport PMEA as well as AZT-monophosphate, and their efflux required adequate levels of intracellular ATP.[55] We showed that this cell line selectively overexpressed MRP4, and further studies using somatic cell genetics, demonstrated dominant resistance to PMEA was conferred by cell fusion with this cell line; moreover, this resistance was directly linked to the transfer of chromosome 13 (the location of MRP4).[55] PMEA resistance provided by MRP4 was also confirmed in subsequent studies by Lee et al[56] using a cell line expressing the human MRP4 cDNA. However, the resistance to PMEA was very small in these studies and may represent either an intrinsic PMEA resistance of the NIH 3T3 and/or poor PMEA drug uptake.

To further assess the role of MRP4 in the acquisition of PMEA resistance, we evaluated MRP4 expression in 2 additional PMEA-resistant cell lines (**Figure 2**). The first cell line was a K562 cell line described by Hatse et al[10] that had impaired PMEA accumulation linked to enhanced "secretion" of PMEA. This accumulation defect was temperature and energy dependent. We also isolated an additional PMEA-resistant T-lymphoblastoid cell line (CEM/PMEA-2) Both of these cell lines strongly overexpressed MRP4, but not MRP1, a finding strongly supporting MRP4's role in the acquisition of resistance to the cytotoxicity of PMEA. The importance of MRP4 to antiretroviral therapy is 2-fold. First, it can decrease the intracellular concentration of the antiretroviral drug (eg, PMEA), which will lead to impaired suppression of HIV replication. Second, high levels of MRP4 protect HIV-infected cells from the cytotoxic effects of antiretroviral drugs (see **Table 1**).[55] The cytostatic activity of purine analogues, PMEA and the aminopurine PMEDAP, was 5- and 26-fold less in the CEM/PMEA-2 cell line compared with the CEM/0. A further evaluation of additional agents such as phosphonoformic acid (PFA) or phosphonoacetic acid revealed only moderate resistance (< 2-fold), which indicates MRP4 requires more than an anionic moiety to recognize its substrates. Furthermore, additional studies demonstrated that HIV replication was less effectively inhibited in cells overexpressing MRP4 (see **Table 2**).[55] For instance, the concentration of drug required to inhibit HIV replication by 50% (EC50) was in-

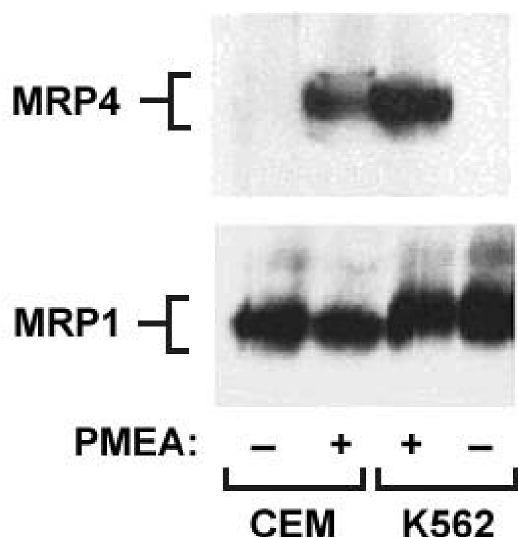

Figure 2. MRP4 but not MRP1 is overexpressed in PMEA resistant cell lines. A total lysate was prepared from the indicated cells and 200 µg of total protein was used for immunoblot analysis using an anti-MRP4 antibody (upper panel) and anti-MRP1 antibody (lower panel). PMEA - and + refer to the parental (CEM/0 or K562/0) and the PMEA selected cell lines (CEM/PMEA-2 or K562/PMEA-1), respectively.

creased 27-, 20-, 6-, 20-, 14- and 18-fold for PMEA, PMEDAP, 5-FPMPA, 5-FPMPDAP, R-PMPA, and R-PMPDAP, respectively, in the MRP4 overexpressing, CEM/PMEA-2 cells. Notably, the resistance to the cytosine dideoxynucleoside analog (ddC) was trivial. Finally, although modest resistance was achieved for guanine analogs, PMEG and 5-FPMPG, the overall findings suggest a preference for adenosine analogs, which is consistent with our previous findings in MRP4 overexpressing cells.[55] Cumulatively, MRP4 overexpression correlates with the acquisition of resistance to these modified nucleoside analogs, which leads to impaired effectiveness in inhibiting HIV replication. It has been previously unknown if the level of MRP4 expression varies among individuals (see below) or if sequence variations exist in MRP4, however, such variation in sequence and level may affect therapeutic response.

The expression of MRP4 may also affect cancer chemotherapy, as suggested in a recent study. Using an MRP4 transfected NIH3T3 cell line, Chen et al[57] demonstrated that MRP4-overexpressing cells were resistant to the cytotoxic effects of 6-mercaptopurine and 6-thioguanine, important drugs in the treatment of childhood leukemias; however, they failed to determine which thiopurine substrate was actually transported. We recently evaluated MRP4 expression by reverse transcriptase polymerase chain reaction (rt-PCR) assays in diagnostic pediatric leukemias and found wide variations in MRP4 expression (**Figure 3** and **Table 3**). It is notable that T-cell leukemias appear to have a somewhat higher level of MRP4 expression than B-cell leukemias (**Table 3**). In addition, MRP4 was undetectable in the small number of acute myeloid leukemias studied. Variable expression for MRP5 was also detected in these leukemias (not shown).

In summary, MRP4 plays a clear role in protection against the cytotoxic effects of purine-derived antiviral and anticancer agents. The results in leukemia lymphoblasts suggest that variable MRP4 and MRP5 expression could play an important role in response to chemotherapy of childhood leukemia. Moreover, interindividual variations in MRP4 expression could also affect the outcome of immunosuppressant therapy with thiopurines.

Recent studies have demonstrated that MRP5 not only transports PMEA but also monophosphate derivatives of thiopurines.[58] The implication of these findings is that MRP5 affects not only antiretroviral therapy but also cancer chemotherapy as well. Because MRP5 was capable of transporting both adenosine and guanosine monophosphate derivatives, Jedlitschky and colleagues[59] reasoned that this transporter may be involved in the efflux of cyclic nucleotides, including cGMP and cAMP, which had been described biochemically for many years. Using membrane vesicle preparations developed from cells transfected with an MRP5 expression vector, they demonstrated that MRP5 is a cGMP transporter with an affinity that closely resembles the cGMP transporter described in erythrocytes by Schultz and colleagues.[37] Moreover, immunoblot analysis revealed that MRP5 is expressed in erythrocytes, and is likely the cGMP transporter previously described in erythrocytes.[36,37] In contrast, MRP5 was discovered to be a rather poor cAMP transporter with a very low affinity for cAMP,[59] and based on this, it was inferred that MRP5 was unlikely to efflux the cellular cAMP. Notably, these authors also demonstrated that clinically important phosphodiesterase inhibitors inhibit cGMP transport. For example, sildenafil (ie, Viagra), a potent phosphodiesterase inhibitor is also a very effective MRP5 inhibitor. Thus, one could speculate the vasodilatory effects of sildenafil could be due to inhibition of MRP5-mediated cyclic nucleotide transport.

Cumulatively, these findings suggest that the balance of cellular nucleotides (both natural and drug) is mediated by molecules such as MRP4 and MRP5.

Table 1. Cytostatic activity of test compounds against wildtype and PMEA-resistant CEM cells[a]

Compound[b]	CEM/0	CEM/PMEA-2(fold resistance)
PMEA	100	>500 (>5-fold)
PMEDAP	14.5	380 (26)
PFA	172	295 (1.7)
PAA	141	195 (1.4)
cAMP	610	305 (0.5)
5-Br-dUrd	7.1	8.7 (1.2)
5-Fl-dUrd	0.006	0.004 (0.7)
araC	0.009	0.007 (0.8)
Methotrexate	0.004	0.006 (1.5)
EICAR	0.360	0.185 (0.5)
Ribavirin	13.5	20 (1.5)
Tubercidin	0.075	0.072 (1.0)

[a] Data expressed as IC_{50} of drug concentration required to inhibit tumor proliferation by 50%.
[b] PMEA, 9-(2-Phosphonylmethoxyethyl)adenine; PFA, phosphonoformic acid (foscarnet); PAA, phosphonoacetic acid; cAMP, 3',5'-cyclic Adenosine monophosphate; 5-Br-dUrd, 5-bromo-2'deoxyuridine' 5-Fl-dUrd, 5-fluoro-2'deoxyuridine; 5-ethynnyl-1-β-D-ribofuranosylimidazole-4-carboxamide; araC, cytosine arabinoside.

Table 2. PMEA-resistant cells have impaired anti-HIV activity for adenine based compounds[a]

Compound	BASE	CEM/0	CEM/PMEA-2 (Fold-resistance)
PMEA[b]	adenine	11 ± 4.0	300 ± 100(27)
PMEDAP	2,6-DAP	2.8 ± 2.0	57 ± 15 (20)
(S)-FPMPA	adenine	13 ± 0.0	82 ± 23 (6)
(S)FPMPDAP	2,6-DAP	8.8 ± 0.91	180 ± 0.0 (20)
(R)PMPA	adenine	4.2 ± 2.4	58 ± 10 (14)
(R)PMPDAP	2,6-DAP	0.76 ± 0.4	14 ± 8.3 (18)
(S)FPMPG	guanine	8.3 ± 1.8	15 ± 6.5 (2)
PMEG	guanine	> 0.8	3.3 ± 0.6 (4)
ddI	inosine	10 ± 0.0	25 ± 13 (2.5)
ddC	cytosine	0.020 ± 0.0	0.026 ± 0.0 (1)
AZT	thymidine	0.005 ± 0.001	0.02 ± 0.0 (4)

[a]Data expressed as 50% effective concentration (EC_{50}) required to inhibit HIV-induced cytopathicity by 50% with the values after +/- indicating the standard deviations.
[b]PMEA, 9-(2-Phosphonylmethoxyethyl)adenine; PMEDAP, 9-(2-Phosphonylmethoxyethyl)-2,6,-diaminopurine; FPMA, 9-(3-fluoro-2-phosphonylmethoxyropyl)adenine; FPMPDAP, 9-(3-fluoro-2-phosphonylmethoxyropyl)- 2,6,-diaminopurine; PMPA, 9-(3-hydroxy-2-phosphonylmethoxyropyl)adenine; PMPDAP, 9-(3-hydroxy-2-phosphonylmethoxyropyl)-2,6,-diaminopurine; FPMPG, 9-(3-fluoro-2-phosphonylmethoxyropyl)guanine; PMEG, 9-(2-phosphonylmethoxyropyl)guanine; ddI, di-deoxyinosine, ddC, di-deoxycytosine; AZT, azidothymidine.

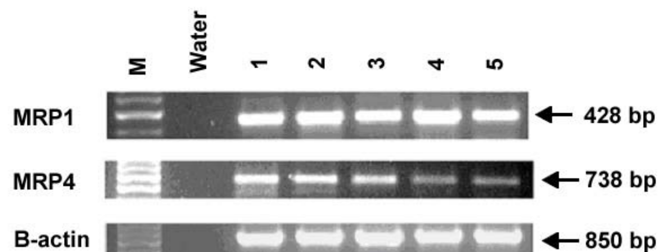

Figure 3. RT-PCR assay of MRPs 1 and 4 in childhood leukemias. cDNAs were reverse transcribed from 1 µg total RNA from 5 childhood acute lymphoblastic leukemia specimens and used to amplify MRPs 1 and 4. Expression of MRPs was normalized to B-actin. Amplicons were detected by electrophoresis on a 2% agarose gel with ethidium bromide.

THE ROLE OF MRP4 AND MRP5 IN NUCLEOTIDE EFFLUX

We evaluated the K562/PMEA and the CEM/PMEA-2 cell lines for changes in gene copy by fluorescence in situ hybridization (FISH) (**Figure 4**). Our previous study demonstrated MRP4 overexpression as a consequence of gene amplification[55]; however, other mechanisms could account for MRP4 upregulation (eg, transcription or mRNA stabilization). The metaphase spreads of K562/PMEA-1 and CEM/PMEA-2 revealed no authentic gene amplification (**Figure 4**). Several possible explanations are likely for MRP4 overexpression. However, it is unlikely that MRP4 overexpression is due to substrate stabilization, because its overexpression persists despite cell growth in the absence of PMEA (not shown). Another possibility is that the MRP4 expressed in these cells is altered and leads to a more stable transcript. The last possibility is that MRP4 transcription is enhanced.

We speculate that MRP4 transcription is controlled by its natural substrates, cyclic nucleotides (**Figure 1**). In support of this idea is the finding that the MRP4 substrate, PMEA, inhibits adenylate cyclase.[60] This inhibition was postulated to decrease the intracellular level of cAMP and may be part of an autoregulartory loop. Thus, based on the relationship between PMEA exposure, its ability to inhibit adenylate cyclase, and upregulation of MRP4, we propose that MRP4 is regulated by intracellular cAMP. Currently, the regulation of MRP4 by cyclic nucleotidesremains uncharacterized but is clearly a subject of future studies.

Our recent study demostrated that hepatic MRP4 is upregulated in response to elevated levels of hepatic bile acids.[61] This occurred following a profound decrease in expression of the major bile acid effluxing transporter sister of P-glycoprotein (SPGP) aka bile salt export protein (BSEP).[61] The decreased SPGP/BSEP was secondary to genetic ablation of its major transcriptional regulator, the nuclear receptor FXR/BAR, a bile acid responsive receptor.[62] As a consequence, a dramatic elevation

Table 3. MRP4 levels vary in pediatric leukemias at diagnosis[a]

	MRP1	MRP4
B-Cell		
1	++	-
2	++	-
4	++	+
5	++	++
6	++	+
8	+++	++
19	+++	-
22	+++	-
24	+++	++
27	+++	++
30	+++	++
31	+++	++
32	+++	++
35	+++	+
36	++	-
39	+++	++
40	+++	-
49	++	++
51	++	-
52	+++	++
53	+++	+
T-Cell		
10	+++	++
11	+++	+++
12	+++	+
20	+++	+++
21	+++	-
23	+++	++
28	+++	++
29	+++	+++
33	+++	++
43	++	-
44	+++	+
45	+++	+
46	+++	+
AML		
15	+++	-
16	++	-
17	++	-

[a] Total RNA was isolated and cDNAs were produced by reverse transcription from 1 µg total RNA. The cDNAs were used to amplify MRPs 1 and 4 and the oligonucleotide primers were: MRP1, 5'-catcaccacgctgcttgct, 5'-gcgcattccttcttccagt-3'; and MRP4, 5'-cgcgtgttcttctggtgg-3', 5'-tggtcctgatcctggcatc-3'. The PCR amplification conditions were 5 min at 94°, followed by 35 cycles of 94° for 30 s, 60° for 45 s, 72° for 1 min, and 72° for 7 min. Expression of MRPs was normalized to β-actin (primers: 5'-atctggcaccacaccttctacaatgagctgcg-3', and 5'-cgtcatactcctgcttgctgatccacatctgc), amplified for 35 cycles of 94° for 30 s, 60° for 45 s, 72° for 1 min, followed by 72° for 7 min. PCR products were size fractionated on a 2% agarose gel and visualized by staining with ethidium bromide. Bands were scored using a scale of undetectable (-) and low (+), intermediate (++), or high levels of product (+++).

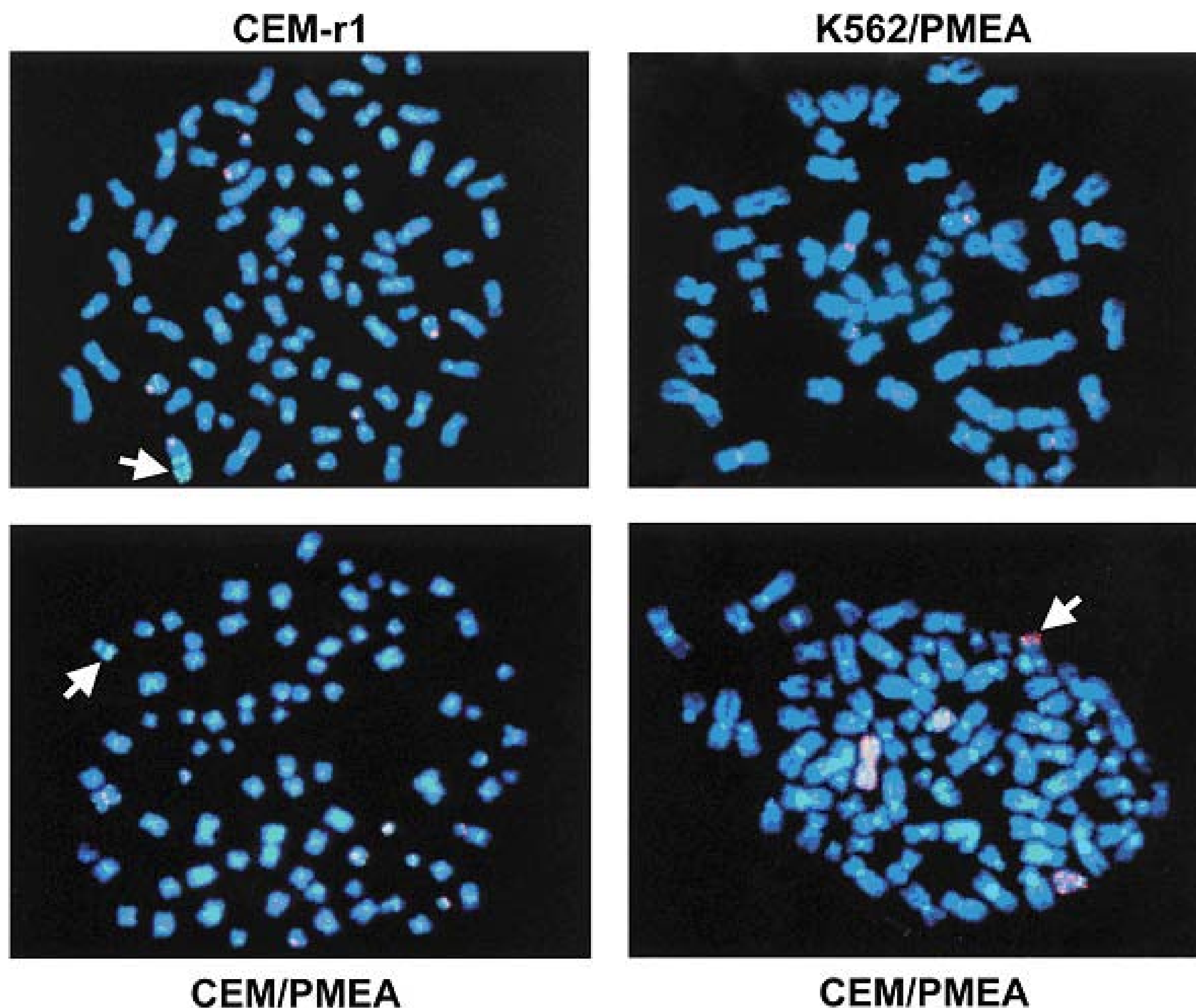

Figure 4. Function and regulation of nucleotide effluxing MRPs. MRP4 and MRP5 are shown on the membrane of the cell. (A) Extracellular signals can activate via their receptors (R) nucleotide cyclases, which lead to an elevation in cyclic nucleotide (cNMP); alternatively nitric oxide (NO) activates a similar pathway leading to the conversion nucleotide monophosphate (NMP) to cyclic nucleotide monophosphate (cNMP). The accumulated cNMP is effluxed out of the cell by either MRP4 and/or MRP5. The energy for the efflux is provided by ATP hydrolysis. We also hypothesize that cNMP may play a role in the transcriptional regulation of MRP4 and/ or 5. (B) High levels of hepatic bile acids can lead to the upregulation of MRP4. We propose that MRP4 activation occurs though bile-acid activation of the orphan nuclear receptor pregnane-X-receptor (PXR) or steroid and xenobiotic receptor (SXR).

of hepatic bile acids occurs. Moreover, bile acids further increase in these FXR/BAR-null mice after cholic acid feeding, and this is closely correlated with a concomitant rise in MRP4 expression.[60] Currently, it is unknown if MRP4 is directly upregulated by bile acids (perhaps by the pregnane-X-receptor/steroid and xenobiotic receptor (PXR/SXR), which we and others have shown are transcriptionally activated by bile acids). However, MRP4's upregulation most likely occurs as a compensatory mechanism to protect the liver from injury caused by bile acid overload (**Figure 4**). Certainly, the ability of MRP4 to protect the liver from bile acid overload will be re-

vealed by an MRP4 knockout animal, which we have developed (unpublished). Finally, it is conceivable that mice nullizygous for PXR/SXR will be unable to upregulate MRP4 in the face of a bile acid challenge.

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