Selecting surfactants for the maximum inhibition of the activity of the multidrug resistance efflux pump transporter, P-glycoprotein: conceptual development.

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Received: 07 October 2010; Accepted: 28 October 2010

ABSTRACT

Amphiphilic excipients, such as surfactants, have been shown to be inhibitors of the multidrug resistance (MDR) efflux pump transporter protein, P-glycoprotein (Pgp). *In vitro* studies using many surfactants have demonstrated that those with an optimum hydrophilic-lipophilic balance (HLB) exhibit greater efflux pump inhibition than those that are either very hydrophobic, or very hydrophilic, although the correlation of HLB to Pgp inhibition activity remains weak. Using the data from multiple *in vitro* studies, a model has been conceptualized that underscores the attributes of both the HLB and the critical micellar concentration (CMC), occurring in tandem, and unable of being varied independently, as key determinants toward prediction of surfactant Pgp inhibition activity. The algorithm that formalizes this concept provides a 'semi-rational' method of choosing surfactants for a specific type of cancer for maximum inhibition of MDR.

KEY WORDS: Surfactant, P-glycoprotein, multidrug resistance, prediction, HLB, CMC, excipient, chemo-resistance

INTRODUCTION

The development of resistance to a broad range of structurally dissimilar anticancer agents is a major cause for the failure of chemotherapy. Chemotherapeutic agents are pumped out of cancer cells by transport proteins resulting in clinical multidrug resistance (MDR). P-glycoprotein (or Pgp or ABCB1) is one of these proteins. It belongs to a group of transporter proteins that are known as efflux pumps and whose genes are encoded by the ATP binding Cassette (ABC) gene family. Its expression in chemo resistant cancers has been shown to correlate with poor chemotherapeutic response and prognosis.

Many excipients, particularly those which possess amphiphilic properties, such as bile salts, phospholipids, surfactants, anionic gums and solubilizing agents have been identified as potent inhibitors of Pgp. Many of these agents have been evaluated *in vitro* for their potential to reverse the multidrug resistance caused by Pgp.

A correlation has been shown to exist between certain molecular characteristics of surfactants such as the density of electron donor and acceptor sites and their capability to function as multidrug resistance (MDR1) substrates (1, 2). The rearrangement (3), redistribution (4) and/or

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depletion (5) of cholesterol in the cell membrane has shown to be associated with modulation of efflux pump function. The cholesterol associative or perturbative activity of surfactants could therefore be considered an important attribute for modulating drug efflux activity. The hydrophilic-lipophilic balance (HLB) and the critical micellar concentration of surfactants has been correlated with the magnitude of their MDR efflux pump modulating activity (6), as has the presence (and number) of specific functional groups on the molecule, such as oxyethylene. The charge shielding ability of ionic surfactants has been speculated to be involved in bypassing the MDR mechanism of drug resistant cells (7). Molar refractivity (8) and hydrophobicity have also been associated with the magnitude of MDR inhibition.

DEVELOPMENT OF THE CONCEPT

Several reports in the literature suggest that surfactants with intermediate lipophilic properties demonstrate optimum modulation of P-glycoprotein drug efflux activity. Pluronics with an intermediate length of the propylene oxide block (from 30 to 60 units) and HLB < 20 were the most effective at inhibiting Pgp efflux in bovine brain microvessel endothelial cells (9). Structure-activity relationship studies of tocopheryl polyethylene glycol succinate (TPGS) analogs, demonstrated that rhodamine 123 permeation rate through Caco-2 cell monolayers was strongly influenced by the polyethylene glycol chain length, with an optimum linear PEG molecular weight between 1000 and 1500 Da (10). Surfactants with HLB values between 10 and 17 were most the effective in enhancing the intracellular accumulation of Epirubicin in Caco-2 cells (11). Within a homologous series of Triton surfactants, maximum disruption of mitochondrial membranes occurred at HLB values between 12.5 and 13.5 (6). The critical micellar concentration (11) (CMC), lipophilicity, steric properties and hydrogen-bonding capacity of surfactants have all been implicated as general requirements for MDR reversal. (12) Each of them appears to be a necessary, but not sufficient, determinant of MDR reversal activity.

Table 1 Physico-chemical attributes of surfactants

Surfactant	Molecular weight, g/mole	Critical micellar concentration, mM	HLB
n-Octylglucoside	292.4	14.5	
Octylphenolpoly(ethyleneglycolether)$_{10}$, [Triton X-100]	647	0.2-0.9	13.5
Polyethylene glycol hydroxystearate [Solutol]	344.5	0.05-0.2	14-16
Tocopheryl polyethylene glycol succinate [TPGS]	1513	0.13-0.33	15-19
Octylphenolpoly(ethyleneglycolether)$_{7}$, [Triton X-114]	537	0.2-0.35	12.4
3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate, [CHAPS]	614.9	6.41	
3-[(3-Cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane-sulfonate, [CHAPSO]	630.88	8	
Ethylene oxide condensates of fatty alcohols, [Lubrol, Brij]	362.6	0.09	9.7
Monolaurylpolyethylene-glycolether-sorbitan, [Tween 20]	1227.5	0.08	16.7
Polyoxyethylated castor oil, [Cremophor EL]	1680	0.12-0.18	'12-14
Dioctyl sodium sulfosuccinate, [Aerosol OT]	444	0.64	10.5
Pluronic F68	8400	0.04	29
Pluronic P85	4600	0.07	16
Tween 80	1310	0.01-0.02	15
Sodium dodecyl sulfate, [SDS]	288.4	6.0-8.0	40
Sodium deoxycholate	414.6	2.0-6.0	24-26
Benzalkonium chloride	354	0.04	
Cetyl trimethyl ammonium bromide, [CTAB]	364.5	0.92	10
Pluronic L81	2750	0.024	2
Pluronic L61	2000	0.1	3
Polyoxyethylene monostearate [Myrj 52]	2047	0.05	15-16.9
Span 80	428.6		4.3
Pluronic F127	12600	0.04	22

The two attributes of CMC and HLB can be used as predictors for the lipophilicity (13), hydrogen bonding capacity and, to a certain extent, the steric properties, of surfactant molecules (at least within a homologous series) (14).

It is therefore not unreasonable to assume that the effectiveness of a particular surfactant as an MDR reversing agent must be an (inverse) function of the difference of its CMC and HLB from certain optimal (i.e. values that result in the greatest MDR inhibition) values of CMC and HLB. Furthermore, precisely because of the demonstration of such optimal (MDR reversing) values in the literature, the function may be assumed to be parabolic in nature, with transporter inhibiting activity decreasing as the values of CMC and HLB deviate from their 'optimum' values.

The function used to arrive at a numerical value of the effectiveness of a surfactant as an MDR reversing agent is:

$$f(HLB,CMC)=1/\{SQRT\{[(HLB-8.5)^2] \times [(CMC-0.045)^2]\}\} \quad 1$$

where, SQRT stands for the square root.

The difference between the HLB and the optimum HLB (and the CMC and the optimum CMC) are squared in order to account for the fact that the HLB or CMC of surfactants may be lesser or greater than their corresponding optimum values. The square root is introduced to negate the 'squared' operation. In effect, therefore, the effectiveness of a particular surfactant as an MDR reversant is calculated to be an inverse function of the difference of its CMC and HLB from the optimum values of CMC and HLB respectively, as elaborated earlier.

These 'optimum values ' of CMC,(C_{opt}) and HLB, (H_{opt}), of 0.045 mM and 8.5 respectively, represent the geometric mean of the CMCs and HLBs of the poly(oxyethylene-poly(oxypropylene) (Pluronic) class of surfactants used in the study from reference 12 (Table 2). While there is no a'priori justification for using these particular values from this particular study, it can be seen that the former spans three orders of magnitude and the latter includes a wide range of HLBs. Another reason for using the data from this study as a 'predictor set', is that the Pluronics are recognized as relatively non-toxic agents and some have progressed to the clinic as MDR inhibitors (15). According to Berthelot, and consistent with London's theory of dispersive forces, the attraction constant between dissimilar substances is represented by the geometric mean of their individual internal attraction constants. Because micelle formation is driven, in part by dispersive forces, the geometric mean of the CMCs' is better representative of the optimal CMC, where maximum transporter inhibition might be expected to occur. Likewise, the HLB of non-ionic surfactants has been shown to correlate with the oil-water partition coefficient and the solubility parameter (16). The solubility parameter term arises as a result of the geometric mean assumption (see above) in the Scatchard-Hildebrand equation (17). Therefore, the geometric mean of the HLBs' of individual surfactants is more likely to yield an 'optimum' HLB at which PgP inhibition might be maximum.

The HLB and CMC of the two surfactants designated 'Fictional' in the last two rows of Table 2 were obtained as follows: From the polynomial regression equation in Figure 1, the maximum value of x (x_{max}) was calculated by applying the function, $f(x_{max}) = -b/2a$, where a and b are coefficients of x^2 and x respectively in the polynomial equation. This value of x_{max} of 3.97 was substituted in the polynomial equation to determine the maximum value of y (the same

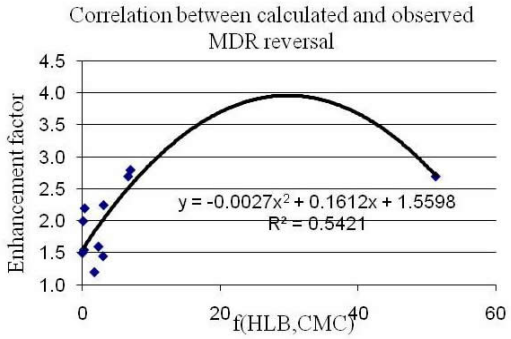

Figure 1 Correlation between the last two columns in Table 2.

as the numerical value of f(HLB, CMC), where the inhibition of the Pgp transporter is expected to be maximum).

The paired values of CMC and HLB of 0.1, 9.12 and 0.04, 1.8 respectively, were determined by iteration of Equation 1 using f(HLB, CMC) = 29.85. Hence, a pluronic surfactant with a CMC of 0.1 mM and an HLB of 9.12 (or one with a CMC of 0.04 mM and an HLB of 1.8) would be expected to maximally accumulate R123 in bovine brain microvessel endothelial cells. The attributes of the surfactants designated 'Fictional' in the tables that follow were obtained similarly.

It should be noted that the data in Tables 2 through 6 (shown in Figures 1-5) was obtained from *in vitro* experiments. The relevance of the CMC, when such formulations are administered intravenously and diluted by blood, is discussed later.

Table 2 The effect of various Pluronics to increase the accumulation of R123 in bovine brain microvessel endothelial cells as presented in reference 12.

Surfactant	CMC, mM	HLB	f(HLB,CMC)	Enhancement factor
F88	0.246	28	0.255	1.5500
F108	0.022	27	2.350	1.6000
F127	0.003	22	1.764	1.2000
L35	5.260	19	0.018	1.5000
P85	0.065	16	6.667	2.7000
L64	0.483	15	0.351	2.2000
L43	2.162	12	0.135	2.0000
P103	0.006	9	51.282	2.7000
L81	0.023	2	6.993	2.8000
L101	0.002	1	3.101	2.2500
L121	0.001	1	3.023	1.4500
Fictional	0.100	9.12	29.851	3.9700
Fictional	0.040	1.8	29.851	3.9700

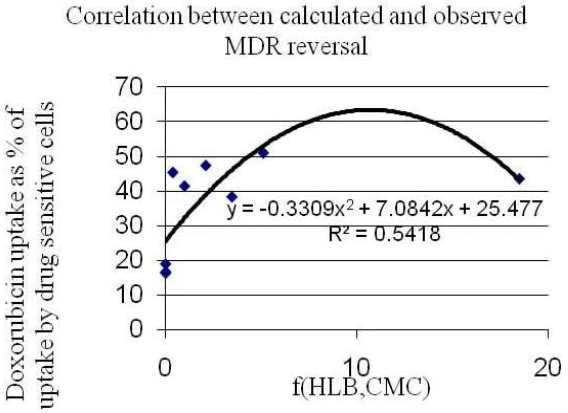

Figure 2 Correlation between the last two columns in Table 3.

Table 3 Uptake of doxorubicin in MDR cells expressed as a percentage of its uptake in drug sensitive cells. Human leukaemic cell line CCRF-CEM and its MDR derivative R100 as presented in reference 9.

Surfactant	CMC, mM	HLB	f(HLB,CMC)	Uptake as percent of drug sensitive cells
n- Octylglucoside	14.500	12.5	0.017	19.0000
Triton X-100	0.550	13.5	0.396	45.3000
Triton X-114	0.300	12.4	1.006	41.4000
CHAPS	6.000	15	0.026	16.7000
CHAPSO	8.000	15	0.019	16.4000
Lubrol	0.090	9.7	18.519	43.5000
Tween 20	0.080	16.7	3.484	38.3000
Cremophor EL	0.150	13	2.116	47.3000
Tween 80	0.015	15	5.128	50.9000
Fictional	0.29	9.12	10.7	63.39
Fictional	0.059	1.8	10.7	63.39

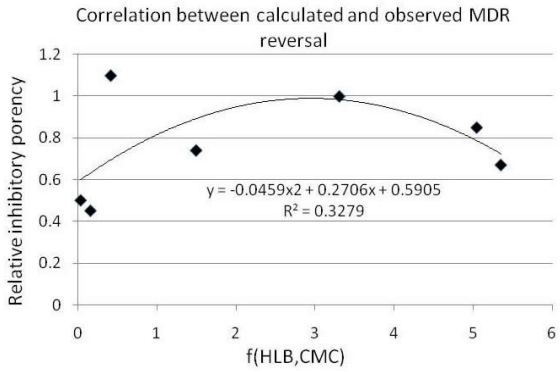

Figure 3 Correlation between the last two columns in Table 4.

Table 4 The relative inhibitory potency to inhibit Rhodamine 123 (R123) efflux (relative to verapamil) for several surfactants in murine monocytic leukemia cells overexpressing P-glycoprotein as presented in reference 10. The CMC of the surfactants listed was measured in Hank's balanced salt solution.

Surfactant	CMC, mM	HLB	f(HLB,CMC)	IP_{rel}
TPGS	0.33	17	0.413	1.1
Cremophor EL	0.179	13.5	1.493	0.74
Pluronic L81	0.0145	2	5.044	0.85
Pluronic L61	0.1	3	3.306	1
Tween 80	0.023	15	5.35	0.67
Sodium dodecyl sulfate	1.04	40	0.032	0.5
Pluronic F68	0.357	29	0.156	0.45
Fictional	1.68	9.12	2.95	0.989
Fictional	0.196	1.8	2.95	0.989

Table 5 Enhancement of intracellular accumulation of epirubicin in Caco-2 cells in the presence of various surfactants as compared to accumulation of epirubicin alone as presented in reference 11. CMC of the surfactants was measured in Dulbecco's modified Eagle medium.

Surfactant	CMC, mM	HLB	f(HLB,CMC)	Enhancement factor
span80	nd	4.3	nd	1.4000
brij30	0.360	9.7	2.646	1.7000
Tween 80	0.240	15	0.789	1.9000
Tween 20	0.270	16.7	0.542	1.9000
Myrj 52	0.310	16.9	0.449	1.8000
Sodium dodecyl sulfate	4.100	40	0.008	1.5000
Fictional	1.15	9.12	1.46	2.06
Fictional	0.15	1.8	1.46	2.06

Table 6 Enhancement of rhodamine-123 by Caco-2 cells using methoxypolyethylene glycol-block-polycaprolactone diblock polymers as presented in reference 18. CMC of the surfactants was measured in 1,6-diphenyl-1,3,5-hexatriene using fluorescence spectroscopy.

Surfactant	CMC, mM	HLB	f(HLB,CMC)	Enhancement factor
MePEG(17)-b-PCL(2)	3.210	16.6	0.039	1.2500
MePEG(17)-b-PCL(5)	0.250	12.2	1.318	3.5000
MePEG(17)-b-PCL(10)	0.005	8.4	251.889	1.6700
MePEG(45)-b-PCL(4)	0.275	18.3	0.444	1.4200
Fictional	0.0575	9.12	126.21	118.82
Fictional	0.0462	1.8	126.21	118.82

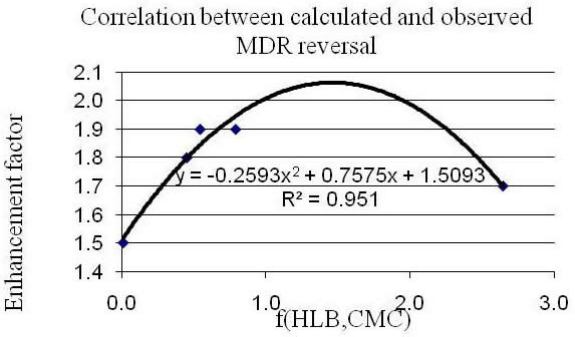

Figure 4 Correlation between the last two columns in Table 5.

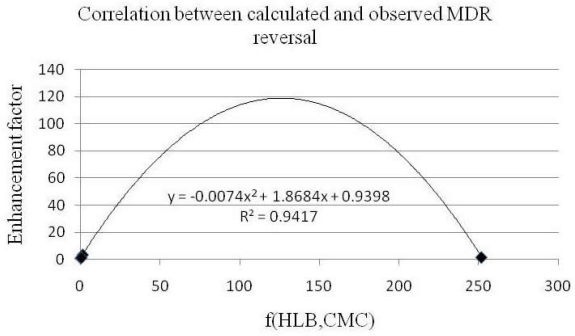

Figure 5 Correlation between the last two columns in Table 6.

The usual obvious caveats apply to the analysis and conclusions arrived at in this paper. The correlation between the HLB and CMC of surfactants generally apply within a given class of structurally similar surfactants. However, in this report, the correlation has been extended to structurally and ionically disparate surfactant classes. Mechanisms (by which Pgp inhibition may occur) that are unrelated to the amphiphilic properties of the surfactants (as related to the disruption or rearrangement of the phospholipid bilayer cell membrane) have not been taken into consideration. Such mechanisms, which may include enzyme or apoptotic modulation (19), ATP depletion (20), glutathione extrusion (21) or mitochondrial reactive oxygen species

modulation (22) may superimpose upon, or indeed, even predominate, the cell membrane modulatory effect or the Pgp substrate effect of these amphiphilic surfactants. The optimum HLB and CMC values for surfactants to demonstrate maximum transporter inhibition have been assumed to be the geometric mean of the values of selected Pluronic surfactants and these 'predictor set' values have been used to predict (HLB and CMC) values that result in maximum inhibition across a variety of structurally diverse surfactants, in different cell cultures, for different classes of chemotherapeutic drugs. Furthermore, the CMC of surfactants used in these different studies have been measured in aqueous media of different compositions or, in one instance, in an organic solvent (Table 6).

The correlation coefficients (> 0.5, except for the data from reference 10, considered to be significant for experiments conducted in biological media) for the curve could be construed as a validation of the optimal HLB/CMC and the parabolic function approach hypothesis for the selection of surfactants. However, it should be remembered that any periodic function can be made a sum of a set of simple oscillating (sine/cosine) functions (23). Hence the relatively high "goodness of fit" for the data may not be a true indicator of the validation of the hypothesis presented in this paper. Furthermore, the lack of experimental points at or around the 'maxima' of the parabolas' lends more uncertainty to the validity of the hypothesis. This is especially evident from Table 6, where the algorithm predicts a Pgp inhibitory effect that is two orders of magnitude greater at the 'optimum value' than that observed with surfactants with HLB, CMC value combinations f(HLB,CMC) removed from the 'optimum'.

In this paper, the relative magnitude of the MDR pump inhibition in a particular study was compared for similar concentrations of the different surfactants, except for the data analysis presented in Table 2 which was obtained from reference 12. The concentrations of individual surfactants where maximal inhibition was observed (for that surfactant) were not used for comparison of their relative MDR inhibition efficiencies, except for Table 2 where only this data was reported in the study. All the analysis is therefore performed (for a particular study) at the same surfactant concentration, except for the analysis in Table 2.

An interesting observation that emerges from this concept is that, for a surfactant with a given HLB, its CMC must assume different values for its transporter inhibition activity to be maximum in different cell cultures (and/or in combinations with different drugs). Such surfactants can be designed by changing the structural attributes of the molecule while making no changes to the ratio of the hydrophilic to hydrophobic regions. In the case of Pluronics, for example, one could introduce branching while keeping the ratio of ethylene oxide (EO) to propylene oxide (PO) monomers constant or by changing the number of EO or PO monomers in selected "blocks" in the polymer (while keeping the total number of EO and PO monomers constant). This observation may also explain, in part, why the literature shows a weak correlation between HLB and efflux pump inhibition. It may very well be that the combined attributes of HLB and CMC must be considered together for efflux pump inhibition prediction, as has been shown in this paper.

Table 7 Calculated blood concentrations of several surfactants

Surfactant	Intravenous administered surfactant concentration[a], mM	CMC (mM), from Table 1
Cremophor EL (Taxol®)	2.23 (0.09[b])	0.12 – 0.18
Pluronic L61 (SP1049C®)	0.015	0.1
Pluronic F127 (SP1049C®)	0.018	0.04
Sodium deoxycholate (Fungizone®)	0.014	2.0-6.0

[a] Assuming a blood volume of 7 liters and a body surface area of 1.8 m^2
[b] The number in brackets represents the concentration of surfactant per hour of infusion time.

Surfactants whose concentration is below their CMC are expected to dissociate (from micellar

structures) into unimers. The mechanism of MDR inhibition by surfactants is not yet completely understood. Hence, there could be instances where unimers dissociated from micelles (below the CMC) may prove more effective (through cell membrane perturbation, for example) in promoting intracellular drug accumulation (24, 25). In other instances, promoting the preservation of micellar architecture in the blood (above the CMC) may prove more conducive to inhibiting MDR.

In the case of the former, i.e. where unimers are necessary for intracellular drug accumulation, it is not immediately evident why the CMC of a surfactant need possess an 'optimum' value so long as an amount of it is administered intravenously that causes its concentration in the blood to not exceed its CMC. This is true assuming that, at no point in its mechanism of action, is either micellar assembly or 'hydrophobic' association with cell membrane components required. For example, while it may be possible that unimers are the predominant species that perturb the structure of the cell membrane, it does not preclude blockage or inhibition events downstream not requiring surfactant micellar assembly or micellar structures. Furthermore, an 'optimum' CMC value may be critical for unimer attachment with, or disruption of detergent resistant membranes such as lipid rafts and caveolae (5) in the cell membrane and the consequent depletion of cholesterol from the cell membrane leading to decreased P-glycoprotein function (4).

Organelle and species specific variation in the structure of the plasma membrane (including differences in the fatty acid profiles constituting the lipid bilayer) implies that the "optimum" CMC values for maximum Pgp inhibition may not be similar for cells derived from different organelles or species. This may account, in part, for the differences in CMC (for a given HLB) that are observed in Tables 2 through 6. Future work that seeks to understand the relationship between the composition of the cell membrane and the 'optimum' CMC for maximum efflux pump inhibition may prove of significant value in this regard.

CONCLUSION

There appears to exist a set of related 'optimum' values of HLB and CMC at which a surfactant may be expected to maximally inhibit the MDR efflux pump for a given cell line in *in vitro* experiments. If true, such a concept improves on existing paradigms with regard to several points:

1. The related attributes of HLB and CMC can be calculated either from existing in vitro data in the literature or from experimentation with related cell lines by using the algorithm described in this report.

2. Recognizing that the attributes of CMC and HLB can only take on a fixed set of values (one cannot be varied independently of the other) and that they are related to one another (for maximum Pgp inhibition to occur).

3. The CMC is an important property of the surfactant and cannot be discounted even when administered blood concentrations of the surfactant are below the CMC.

4. Non-toxic macromolecular surfactants can be designed by structurally changing the attributes of existing ones to meet the 'optimum' CMC/HLB criterion described above.

5. A 'semi-rational' method of choosing surfactants for a specific type of cancer may be facilitated by this concept.

More attention should be focused on the concomitant administration of existing FDA approved surfactants with chemotherapeutic agents as an approach to reverse MDR. This approach is likely to be more cost effective and result in a shorter time to market than, an approach that, (say) involves identification and synthesis of a new chemical moiety as an MDR

inhibitor or via structural alteration of existing chemotherapeutic drugs to enable them to bypass the MDR mechanism. Existing excipient surfactants need to be subjected to more analysis (such as is attempted in this manuscript) and empirical research that will enable effective MDR inhibition while minimizing cytotoxicity.

Notwithstanding all the caveats mentioned above, it is hoped that the empirical model presented in this paper, although imperfect, will serve as an impetus for the development of better 'working hypotheses', and eventually to a model based on first principles. Knowing the optimal CMC and HLB, medicinal chemists should be able to design and manufacture surfactants, or mix and match existing ones, that will be maximally effective as MDR reversing agents, thereby solving one of the more insidious and challenging problems of the failure of chemotherapy today.

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