Physical and biological characteristics of multi drug resistance (MDR): An integral approach considering pH and drug resistance in cancer.

Ziad Omran[†], Paula Scaife[††], Simon Stewart[†††] and Cyril Rauch[*]

[*]School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, College Road, Sutton Bonington, Leicestershire LE12 5RD, UK.

[†]College of Pharmacy, Umm Al-Qura University, Al-Abidiyya, 21955 Makkah, Kingdom of Saudi Arabia

[††]Child Health, Obstetrics and Gynaecology, School of Medicine, University of Nottingham, Maternity Unit, City Hospital Nottingham, Nottingham NG7 1PB.

[†††] School of Social, Historical and Literary Studies, University of Portsmouth, Burnaby Road, Portsmouth PO1 3AS, UK

Corresponding author:

Email: Cyril.rauch@nottingham.ac.uk

Tel: 0044 (0)115 9516451

Fax: 0044 (0)115 95 16 440

20   Abstract

21   The role of the Warburg effect in cancer remains to be elucidated with a resurgence in research

22   efforts over the past decade. Why a cancer cell would prefer to use energy inefficient

23   glycolysis, leading to an alteration of pH both inside and outside of the cell, remains to be

24   uncovered. The development of MDR represents a major challenge in the treatment of cancer

25   and it is explained, so far, by the over expression of drug transporters such as the well-known

26   and archetypal P-glycoprotein (Pgp). However, controversies exist regarding the function of

27   Pgp in multi-drug resistance. We suggest here that Pgp-mediated MDR relies fundamentally

28   on pH alterations mediated by the Warburg effect. Furthermore, we propose that the use of

29   proton pump and/or transporters inhibitors (PPIs/PTIs) in cancer are key to controlling both

30   MDR, i.e. sensitize tumors to antineoplastic agents, and drug-related adverse effects.

31

A lost connection between research fields

Over time, fields of scientific research gain autonomy in proportion to the extent to which they have been freed from economic necessity [1]. They develop their own laws and logics which become field-specific and very often run contrary to those in surrounding fields. They develop increasingly specialized research programmes and these can lead to great achievements. As the classical German social theorist Max Weber observed, 'only by strict specialization can the scientific worker become fully conscious, for once and perhaps never again in his lifetime, that he has achieved something that will endure' [2]. However, Weber also saw the melancholy aspect of ultra-specialization: it leads to the development of research fields that are incommensurable and between which communication is increasingly difficult. It also leads to scientists 'putting the blinkers on' in relation to developments outside their areas of expertise. For example, the somatic mutation theory of cancer together with the "war on cancer" have paved the way to great achievements in molecular biology (e.g. genome project) but their applications to medicine, i.e. oncology, remain minimal since the "magic bullet", i.e. the one gene mutated − one drug concept, that was initially promised is still missing. The constant refining process that accompanies ultra-specialization in scientific fields is comparable to that which occurred in the field of abstract art where, through a process of purification that gradually isolated it from all reference to the wider social world, it became almost entirely propelled by its own inner dialectic [3]. We see here that in its 'purified' state, a field becomes inward-looking.

The results of specialization can be seen in the sub-field of research on MDR in cancer, which suffers from an inherent fundamental paradox. As early as 1973 the drug efflux hypothesis was suggested by Dano Keld [4], which was reinforced in 1976 when Juliano and Ling discovered Pgp in multi drug resistant cells [5]. Since then many works have been carried out to understand the function of Pgp in MDR. However the single use of Pgp to explain MDR in cancer is flawed

as Pgp violates the law of enzyme affinity/specificity on which the entire field of molecular biology is built: '*MDR protein is a very unusual enzyme with extraordinarily broad substrate recognition capabilities; that is, it violates the law of enzyme specificity*' [6]. What is staggering is that even with the presence of a true scientific paradox in Pgp-mediated MDR in cancer, a range of stakeholders, whether economic and market-oriented (Big Pharma), institutional (academia, research organizations) or political (government, pressure groups), have shared (for most are still sharing) many of the same presuppositions about the problem of MDR in cancer and how it might be combatted, although rare attempts exist to suggest changes of strategy in the field of Pgp-mediated MDR [7].

Why is this so? As scientists we know it, because specialist research fields tend to engender in scientists who have been trained in the field, and are thus attuned to its logic, an implicit sense of what is the correct way of doing science and this can inhibit them from gaining insights from other fields [8].

No one would contest the existence of drug efflux mediated by membrane pumps. The question is simply that if membrane pumps exist in MDR cells, how can they work while, at the same time, violating the law of enzyme specificity? Is it really drug transporters that are important or have we overlooked essential components in multi drug resistant cancer cells?

When faced with an apparent paradox it is essential to step out from the discipline and research around how similar issues are dealt with in other fields of enquiry. Understanding the importance of pharmacokinetic / drug delivery is essential to uncover how a drug may or may not cross the bilayer membrane of MDR cells.

To explore the existing connections between MDR in cancer and other fields one will start by recalling concepts used in the field of pharmacokinetics that deals with similar barriers constituted by ATP-ase drug transporters. We shall see that in this context, the Big Pharma industry have focused on determining the optimal biophysical properties of drugs to cross those

82   barriers (irrespective of drug transporters). Next we shall investigate how those biophysical

83   properties emerge by a clearer understanding of membrane physics. This will allow one to

84   underline a number of studies that have emphasized the important role of the membrane in

85   MDR in cancer. We shall then explain how the notion of specificity or affinity is not required

86   as far as Pgp is involved. Finally, one will demonstrate how the Warburg effect and related

87   changes in pH are involved in changing the membrane in such a way to sustain Pgp activity

88   and MDR.

89   In conclusion we will discuss about the role of proton pump inhibitors (PPIs) and membrane-

90   bound proton transport inhibitors (PTIs) to circumvent MDR and improve drug efficacy in

91   cancer.

92

93   **The notion of pharmacokinetics and how it can help in understanding the MDR paradox**

94   The field of pharmacokinetics deals with how drug chemicals are dealt with by complex body

95   systems and as a result how drug chemicals reach their targets. Defining the drug transporters

96   that "cover" biological barriers has been essential for the success of the pharmaceutical

97   industry. The main difference between the field of molecular oncology and pharmacokinetics

98   is that the former works with simple systems (molecules and cells) whereas the later deals with

99   complex body systems. Looking at how the Big Pharma has dealt with biological barriers may

100   yield novel findings that could help to further define MDR.

101   The' 90s were gloomy years for the pharmaceutical industry with productivity falling below

102   expectations and an average innovation deficit of ~1.3-1.8 for new chemical entities per year

103   [9]. During this period these companies adopted approaches that relied on retrieval of

104   information to determine if a chemical would make a 'likely' drug in advance of costly clinical

105   trials. To this end, Lipinski and collaborators [10] produced a set of rules that attempted to

106   identify the best statistical physico-chemical properties required for an oral compound to

107 achieve maximum bioavailability, i.e. to cross all biological barriers (where drug transporters

108 are present) before reaching its target. The first of Lipinski's rules is based on the lipophilic

109 index of the drug, the second on the drug's molecular weight (abbreviated "MW" in the

110 remaining text) and the third and fourth rules concern the drug's electrostatic charge properties.

111 These rules are now established drug discovery paradigms and have been largely embraced by

112 the pharmaceutical industry. Of the four rules, the second (MW<500) stands out by way of its

113 apparent simplicity, being unrelated to complex physico-chemical properties of a drug (as is

114 the charge or lipophilic index) but governed solely by a drug's size or volume. This simplicity

115 infers that basic mechanics apply when drugs cross membranes, cells, tissues and biological

116 barriers.

117 What is worth considering are the following points: (i) The Big Pharma did not focused on

118 drug transporters and Linpinski's rules do not mention drug transporter expression levels when

119 barriers to drugs are considered and; (ii) the drug volume and thus some mechanical properties

120 needs to be considered when drugs cross complex biological barriers.

121 The next question is why and how biophysics is involved in drug efficacy?

122

123 **Why is the drug MW so important to cross barriers? An introduction to the biophysics**

124 **of drug-membrane interactions.**

125 To be (bio)available, drugs must traverse cellular barriers – usually the epithelium or

126 endothelium (e.g. of the gastrointestinal tract, renal tubules or the blood-brain and blood-

127 placenta barriers). To traverse cellular barriers, drugs must cross lipid membranes, and for this

128 Lipinski's 2nd rule postulates that drugs must have a MW<500**.** Therefore, in the sum of

129 energies making up the total activation energy required for a drug to cross cellular membranes

130 a term must exist to underline the role of the membrane. In this case, i.e. when the plasma

131 membrane is considered as a flat object, the physical parameter that best fits such an interaction

132 is the membrane leaflets' surface tension (σ and unit $[\sigma] = N/m$)[1]. Of course the surface

133 tension parameter needs a proper definition especially in cells. All lipids are amphipathic

134 molecules and as a result optimize their individual surface area in membrane leaflet. This

135 optimization results from the energy balance between steric and/or electrostatic repulsion(s)

136 (related to lipids' head) and the lipid contact with water (related to the hydrophobic aliphatic

137 chain(s)). This balance defines the surface tension. Now, when a drug enters a membrane leaflet

138 it will have to "squeeze in" and compress the lipids of the leaflet, namely change the surface

139 tension. This impact on the energy balance of lipids composing the leaflet will have a tendency

140 to repulse, i.e. push out, the drug from the leaflet. However this process is not totally rigid as

141 otherwise chemicals would never cross membranes. In fact, lipids are not static as the thermal

142 agitation exists which allows for some flexibility. So if a small enough chemical incorporates

143 into the leaflet and perturbs it in such a way that the resulting membrane energy is below the

144 ambient thermal energy, then the lipids composing the leaflets will not "feel" any difference

145 between the thermal agitation and the incorporation of the drug. So a drug can incorporate a

146 membrane leaflet if it is small enough.

147 Dimensionally speaking, it follows that a critical cross section for the drug (a_c) can be defined

148 simply by: $a_c \sim k_B T / \sigma$, where $k_B T$ is the thermal energy (k_B is Boltzmann's constant and

149 T the absolute temperature). If the cross section of a drug is lower than the critical value it will

150 incorporate and cross the membrane leaflet, but if it is higher the drug will be blocked.

151 In bilayer membranes, two types of membrane surface tension can be distinguished, the mean

152 surface tension noted σ_0, which corresponds to the sum of the individual leaflet's surface

---

[1] Thermodynamically speaking, the physical parameters that are related to spatial dimensions (namely, volume (V), cross section area (a) or line (r)) are the pressure "P": $\delta E = -P.\delta V$, the surface tension "σ": $\delta E = \sigma.\delta a$, and the tension line "$\gamma$": $\delta E = \gamma.\delta r$. "$\delta$" is the differential operator and "E" the energy. As far as a membrane is considered, it is the surface tension (and thus the cross section area of the drug) that best describes the mechanical (i.e. physical) interaction and is deduced by posing $\delta E \sim k_B T$.

153 tension, and the difference in surface tensions $\Delta\sigma$, which corresponds to the difference

154 between individual leaflet's surface tension. Using optical techniques, M. Sheetz and his

155 collaborators have demonstrated that cells have a large reservoir of membrane [11] and an

156 average membrane tension that is remarkably low ($\sigma_0 \sim 0.003mN/m$) [12]. On the other hand,

157 the difference in surface tensions between leaflets has been demonstrated to be much higher

158 $\Delta\sigma \sim 0.9mN/m$ [13]. Accordingly, and given the magnitude of this parameter, it is more

159 likely to be involved in impairing the transverse movement of chemicals. The previous

160 equation can thus be refined as follows: $a_c \sim k_B T / \Delta\sigma$. Dealing with a parameter as $\Delta\sigma$ is

161 not intuitive and the last equation needs to be resolved physiologically. A fundamental aspect

162 of the difference in surface tension corresponds to its role in pinocytosis associated with the

163 role of specific lipid flippases maintaining the membrane lipid asymmetry [14]. A direct

164 consequence associated with this asymmetry is a more highly packed inner leaflet as it contains

165 more phospholipids than the outer leaflet resulting in the difference in surface tensions (

166 $\Delta\sigma = \sigma_{out} - \sigma_{int} \sim 0.9mN/m$) between the inner (cytosolic) and outer leaflets of the cell

167 plasma membrane. Naturally, bilayer membranes are soft objects and as such, will attempt to

168 release this stored energy. Accordingly, it has been demonstrated that lipid asymmetry

169 corresponds to the physiological motor force that triggers membrane budding, leading to

170 endocytosis (Figure 1) [13, 15, 16]. It is therefore possible to demonstrate that the vesicle radius

171 is written as [13]: $R = 8k_c / h\Delta\sigma$; where k_c is the cell membrane bending modulus and h the

172 membrane thickness. As for drugs small enough that their MW is proportional to their Van der

173 Walls' volume (expressed in $\dot{A}^3$), i.e. $MW \sim V \sim a^{3/2}$, a critical MW (MW_c) can be

174 determined given by:

175 $MW_c = (4/3\sqrt{\pi})(hRk_B T / 8k_c)^{3/2}$ 　　　　　　　　　　　　　　　(Eq.1)

176 The later relation provides a law with regard to the drugs size (or MW) selectivity on their

177 permeation across cellular membranes: $MW_c \cong 240 - 250$ at 37°C [17]. As the MW cut off

178 defined by Lipinski's 2nd rule, i.e. $MW_c = 500$, describes the 90th percentile; the former value

179 (i.e. $MW_c \cong 240 - 250$) is an average in line with Lipinski's rule. Two other important results

180 follow. The first one is that it is also possible to demonstrate that the kinetics of membrane

181 endocytosis is inversely proportional to the vesicle radius [18], i.e.:

182 $k_{endo} \sim 1/R$ (Eq.2)

183 And that the kinetics of transverse movement across the membrane is [17]:

184 $k_{Drug} \sim \exp\left(A \times MW^{\frac{2}{3}} \times k_{Endo} \right)$ (Eq.3)

185 Where, A, is a constant. It does seem that Lipinski's 2nd rule can be explained by considering

186 simple biophysical arguments and that the membrane plays a key role in this process. But what

187 about drug resistant cancer cells?

188

189 **Are alterations in the cell membrane observed in MDR and is the drug MW important in**

190 **multi drug resistant cells?**

191 From what was seen above, if the drug MW is important it is because the membrane is also

192 involved. So changes in the lipid membrane composition and membrane recycling should be

193 expected in drug resistant cells and this seems to clearly be the case. Different studies have

194 reported changes in membrane composition including neutral lipids, phospholipids, cholesterol

195 and fatty acids [19-24], in some cases related to a change in the lipid metabolism of drug

196 resistant cells [22, 25, 26]. This point has been particularly well underlined when the lipid

197 profile of released exosomes was analysed [27]. Also, ultrastructure studies have revealed an

198 increased density of small and large membrane organelles [22, 28-32] and an increase in the

199 kinetics of membrane endocytosis or membrane recycling [29-31, 33, 34] in drug resistant

9

200 cells. It is noteworthy that the release of exosomes is also involved in MDR [35].What is

201 perhaps more important is that the MW of a drug itself was also underlined very early (in 1970)

202 in MDR studies in line with the role that the membrane has in delaying a chemicals influx [36,

203 37]. It is worth noting here that the role of a drugs MW was underlined prior to the discovery

204 of Pgp by Juliano and Ling in 1976 [5]. The connection between membrane endocytosis and

205 the size of a drug chemical with passive influx/uptake of drugs into cells is given by the set of

206 equations described above.

207 The data points clearly to the membrane as a strong effector of drug resistance but why would

208 the membrane be so central when drug transporting is involved in MDR?

209

210 **Drug-membrane biophysical interactions to resolve the multi specificity of drug**

211 **transporters**

212 It is very often suggested that drug transporters work similarly to enzymes in line with the

213 notion of affinity, namely that a drug needs to interact with a transporter to activate the

214 transporter and be expelled. However this view does not work for at least three reasons when

215 focusing on Pgp: (i) the ATP concentration in cells is usually 3-5mM that always exceeds the

216 affinity of Pgp for ATP (K_{mATP}~0.3-1mM) [38, 39], suggesting that the transporter is always

217 "active". (ii) Pgp ATPase activity is relatively independent of the presence of drugs [40], and

218 the affinity of drugs toward transporters is chiefly dependent on their affinity toward the

219 membrane [41]. Finally (iii), the apparent stoichiometry of the hypothesized ATP-coupled

220 active drug transport, i.e. the number of ATP molecules hydrolyzed per drug transported, can

221 be enormous (calculated to be up to ~36000ATP/drug in reconstituted proteo-liposomes) [6,

222 38]. This suggests that while consuming ATP Pgp does not necessarily lead to drug extrusion.

223 Due to the fact that similar conclusions cannot be drawn for drug transporters other than Pgp

224 due to lack of experimental observations, Pgp remains the archetypal transporter involved in

225 MDR and it is believed that Pgp is very likely continuously recycling between "open" and

226 "closed" states by over-consuming ATP. This may explain why Pgp and drug resistance are so

227 sensitive to cellular metabolism [42]. It is interesting to note that Pgp activity leads to a parallel

228 acidification of the extracellular medium [43] that, in turn, is thought to be related to initial

229 metastatic steps [44]. Given that the vast majority of metastatic tumours are also multi drug

230 resistant [45], the recycling between open and closed conformations is likely to be essential to

231 explain the *multi* of drug resistance [46].

232 Here comes an essential point. If Pgp switches between open and closed conformations

233 independently of drugs, what is essential in MDR is that for drugs to be expelled they must

234 remain in the membrane long enough to encounter (or collide with) Pgp. From Eq.3 the kinetics

235 of drug transverse movement is modulated exponentially by two physical parameters related

236 to the biophysical state of the membrane involving the size of the drug (see above) and the

237 kinetics of endocytosis (see below). An increase in the kinetics of membrane endocytosis

238 supporting Pgp function is possible if the Warburg effect and relatively high cytosolic pH are

239 considered.

240

241 **Cytosolic pH, endocytosis and MDR**

242 Regardless of their origin and genetic background cancer cells and tissues have been found to

243 display an abnormality called "proton reversal" which describes the state by which a cell

244 consists of an interstitial acidic microenvironment secondary to an initial, specific and

245 etiopathogenic intracellular alkalosis [47-53]. A failure to induce intracellular acidification and

246 reverse this phenomenon in cancer tissues has been proposed to be the main factor underlying

247 drug resistance including resistance to the induction of therapeutic apoptosis [54-58]. Also,

248 because inner leaflet lipids bear protonable polar heads, pH changes will modify their net

249 charge. In turn this will impact on the sum of electrostatic repulsions and modify membrane

250   difference in surface tension (i.e. decrease the size of pinocytic vesicles and as a result increase

251   the kinetics of endocytosis) [59].

252   To consider any effect of the cytosolic pH on lipid packing it is central to understand the notion

253   of packing from a physics standpoint. At a constant membrane surface area, the lipid packing

254   is given by the optimal area per lipid in the cell membrane. The latter is deduced from the

255   balance between repulsions that occur mostly through electrostatic effects on the polar heads,

256   and attractions, which concern more the hydrophobic and geometric effects that take place

257   between the aliphatic chain(s). Any changes in this balance are expected to affect the optimal

258   area per lipid (i.e. their packing) and membrane shape. As a non-negligible fraction of the inner

259   leaflet consists of negatively charged lipids, such as phosphatidylserine or PIP_2, for example

260   [60] a slight increase in proton concentration around neutrality (e.g. decrease in cytosolic pH)

261   will eliminate or shield these negative charges and decrease the electrostatic repulsion between

262   polar groups. Although such an electrostatic counterion effect might in principle be generalized

263   to intracellular cations, it is obvious that exchangeable protons will have a more pronounced

264   effect on negatively charged lipids. As a final result, a low cytosolic pH is more likely to be

265   central in abolishing the physical repulsion between lipids, and thus decreases the surface

266   tension (i.e. the lipid packing of the cytosolic leaflet - note that both lipid packing and surface

267   tension are proportional to each other). Such a relationship between free electrolytes and the

268   cross section area per lipid in model biomembranes is well known experimentally [61-63]. A

269   similar result was also obtained on living cells [64]. Conversely, when the cytosolic pH

270   increases (i.e. when cells become reliant on the Warburg effect), fewer positive charges will be

271   available to mask the lipids charge, which in turn is expected to increase their repulsions and

272   thus their packing. Thus, this higher lipid packing would increase the surface tension of the

273   leaflet in contact with the milieu of elevated cellular pH in the case of drug resistant cells. So,

274   if the pH affects the packing of lipids, and the packing of lipids affects the intracellular

275   accumulation of drugs, it follows that the cytosolic pH should affect the intracellular

276   accumulation of those drugs. As a result, the changes in cytosolic pH observed when cells

277   switch their state of resistance is an important clue for understanding the observed alterations

278   of intracellular accumulation of drugs as a function of their size. This way of thinking has

279   permitted the theoretical corroboration of the connection between the cytosolic pH (linked to

280   Warburg effect), the membrane biophysical properties and the MDR levels in several cell types

281   [59] (see figure 2). The interaction between the membrane and the cytosolic pH can explain

282   why PPIs overcome the Pgp-mediated MDR [65].

283

284   **Beyond the cell membrane**

285   Using arguments and results developed by us and others the general view is that drug sensitivity

286   or drug resistance can only be understood if one steps outside of a Pgp-centred view to engage

287   with a holistic approach of cancer. This true and fundamental scientific approach is equivalent

288   of saying that what has been exposed in this review needs to be duly criticized as well to push

289   the boundary that it creates under the form of a new research field. In the context of drug

290   sensitivity (or drug resistance or drug refractoriness) in cancer it is essential to underline the

291   fact that many interactions between the various cellular compartments exist that underlines the

292   complexity of the disease that, in turn, may provide fundamental clues as to how MDR

293   progresses. An illuminating study performed in resinless ultrathin EM sections has shown that

294   a staggering network of interconnected cytoskeletal filaments does exist between

295   polyribosomes, mitochondria and a myriad of unidentified small structures attached to the

296   cytoskeleton [66]. Using the same technique, the nuclear space appears as a complex network

297   of core filaments connecting with the nuclear lamina, and the chromosomes appear attached to

298   spindle fibers, which are in turn interconnected through several thin filaments. None of these

299   structures are visible using conventional resin embedding technique. This introduces the

300 concept that the cell has to be considered as a whole, and that this whole is not entirely known

301 also because of the compartmentalization of the research approaches; and this is true for MDR

302 as well. In general the membrane to cytoskeleton connection is entirely deranged in cancer

303 cells, determining an aberrant cell polarization in turn related to the metastatic behaviour [67].

304 Research has been carried out showing that Pgp is linked to actin through ERM and that this

305 connection is key for MDR in human tumor cells [68, 69]. How such interaction can be

306 understood in the framework provided by the membrane is unclear but it underlines that fact

307 that cells should be considered in a holistic way, also because cancer cells are independent and

308 behave as an unicellular microorganism committed to survive in a very hostile environment

309 [70].

310

311 **Conclusion: From bench to bedside**

312 While MDR remains linked to drug transporters, alterations in pH gradient resulting from the

313 Warburg effect across the cell membrane or organelles is well known to impact on the

314 biophysical properties of the cancer cell membrane sustaining drug transporter activity.

315 Therefore it is in theory possible to improve drug uptake by cells by normalizing the pH using

316 PPIs. This point was demonstrated recently in tumor sarcospheres [71]. Furthermore the same

317 study demonstrated that tumor sarcospheres were becoming more sensitive to lower drug doses

318 of anticancer agents raising hope that adverse effects linked to the administration of

319 chemotherapy could, one day, be reduced or controlled in patients [71]. PPIs are amongst the

320 most commonly prescribed drugs in human medicine and have gone through the process of

321 rigorous safety testing and monitoring. Very few clinical side effects are seen even at higher

322 doses and as such it seems easy to justify the continued investigation into the use of this class

323 of drug for the treatment of cancer in companion animals [71-74] and humans [75-78]. They

324 may provide an alternative or additional source of therapy to animals and humans which could

325   result in lower treatment costs, greater availability and safer handling compared to current

326   cytotoxic protocols. PPIs and PTIs could potentially form part of a universal treatment which

327   may have direct benefits in treating a number of different cancer types while combating

328   problems associated with chemotherapy such as drug resistance, severe side-effects and even

329   death secondary to present day chemotherapy.

330

333 Bibliography

334 [1] P. Bourdieu, Science of Science and Reflexivity. , Cambridge: Polity Press. , 2001.
335 [2] M. Weber, Science as a Vocation. , London: Routledge., 1918.
336 [3] P. Bourdieu, The Field of Cultural Production: Essays on Art and Literature. ,
337 Cambridge: Polity Press. , 1983.
338 [4] K. Dano, Active outward transport of daunomycin in resistant Ehrlich ascites tumor
339 cells, Biochim Biophys Acta, 323 (1973) 466-483.
340 [5] R.L. Juliano, V. Ling, A surface glycoprotein modulating drug permeability in Chinese
341 hamster ovary cell mutants, Biochim Biophys Acta, 455 (1976) 152-162.
342 [6] P.D. Roepe, What is the precise role of human MDR 1 protein in chemotherapeutic drug
343 resistance?, Curr Pharm Des, 6 (2000) 241-260.
344 [7] R. Callaghan, F. Luk, M. Bebawy, Inhibition of the multidrug resistance P-glycoprotein:
345 time for a change of strategy?, Drug metabolism and disposition: the biological fate of
346 chemicals, 42 (2014) 623-631.
347 [8] P. Bourdieu, Pascalian Meditations, Cambridge: Polity Press. , 2000.
348 [9] J. Drews, Stategic trends in the drug industry, Drug discovery today, 8 (2003) 411-
349 420.
350 [10] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and
351 computational approaches to estimate solubility and permeability in drug discovery and
352 development settings, Advanced drug delivery reviews, 46 (2001) 3-26.
353 [11] D. Raucher, M.P. Sheetz, Characteristics of a membrane reservoir buffering
354 membrane tension, Biophysical journal, 77 (1999) 1992-2002.
355 [12] F.M. Hochmuth, J.Y. Shao, J. Dai, M.P. Sheetz, Deformation and flow of membrane
356 into tethers extracted from neuronal growth cones, Biophysical journal, 70 (1996) 358-
357 369.
358 [13] C. Rauch, E. Farge, Endocytosis switch controlled by transmembrane osmotic
359 pressure and phospholipid number asymmetry, Biophys J, 78 (2000) 3036-3047.
360 [14] M. Seigneuret, P.F. Devaux, ATP-dependent asymmetric distribution of spin-labeled
361 phospholipids in the erythrocyte membrane: relation to shape changes, Proceedings of the
362 National Academy of Sciences of the United States of America, 81 (1984) 3751-3755.
363 [15] E. Farge, D.M. Ojcius, A. Subtil, A. Dautry-Varsat, Enhancement of endocytosis due
364 to aminophospholipid transport across the plasma membrane of living cells, The American
365 journal of physiology, 276 (1999) C725-733.
366 [16] E. Farge, Increased vesicle endocytosis due to an increase in the plasma membrane
367 phosphatidylserine concentration, Biophysical journal, 69 (1995) 2501-2506.
368 [17] C. Rauch, A. Pluen, Multi drug resistance-dependent "vacuum cleaner" functionality
369 potentially driven by the interactions between endocytosis, drug size and Pgp-like
370 transporters surface density, European biophysics journal : EBJ, 36 (2007) 121-131.
371 [18] C. Rauch, A. Pluen, N. Foster, P. Loughna, A. Mobasheri, D. Lagadic-Gossmann, L.
372 Counillon, On some aspects of the thermodynamic of membrane recycling mediated by
373 fluid phase endocytosis: evaluation of published data and perspectives, Cell biochemistry
374 and biophysics, 56 (2010) 73-90.
375 [19] A. Ramu, D. Glaubiger, H. Weintraub, Differences in lipid composition of doxorubicin-
376 sensitive and -resistant P388 cells, Cancer treatment reports, 68 (1984) 637-641.
377 [20] H. Tapiero, Z. Mishal, M. Wioland, A. Silber, A. Fourcade, G. Zwingelstein, Changes
378 in biophysical parameters and in phospholipid composition associated with resistance to
379 doxorubicin, Anticancer research, 6 (1986) 649-652.
380 [21] L.C. Wright, M. Dyne, K.T. Holmes, C.E. Mountford, Phospholipid and ether linked
381 phospholipid content alter with cellular resistance to vinblastine, Biochemical and
382 biophysical research communications, 133 (1985) 539-545.
383 [22] P.J. Wilder, D.K. Overman, T.C. Tenenholz, P.L. Gutierrez, Differences in myristic acid
384 synthesis and in metabolic rate for P388 cells resistant to doxorubicin, Journal of lipid
385 research, 31 (1990) 1973-1982.
386 [23] A. Mazzoni, F. Trave, Cytoplasmic membrane cholesterol and doxorubicin cytotoxicity
387 in drug-sensitive and multidrug-resistant human ovarian cancer cells, Oncology research,
388 5 (1993) 75-82.

[24] J. Pallares-Trujillo, C. Domenech, M.R. Grau-Oliete, M.P. Rivera-Fillat, Role of cell cholesterol in modulating vincristine uptake and resistance, International journal of cancer, 55 (1993) 667-671.

[25] Z. Kiss, M. Tomono, W.B. Anderson, Phorbol ester selectively stimulates the phospholipase D-mediated hydrolysis of phosphatidylethanolamine in multidrug-resistant MCF-7 human breast carcinoma cells, Biochem J, 302 (Pt 3) (1994) 649-654.

[26] Y. Lavie, H.T. Cao, S.L. Bursten, A.E. Giuliano, M.C. Cabot, Accumulation of glucosylceramides in multidrug-resistant cancer cells, Journal of Biological Chemistry, 271 (1996) 19530-19536.

[27] I. Parolini, C. Federici, C. Raggi, L. Lugini, S. Palleschi, A. De Milito, C. Coscia, E. Iessi, M. Logozzi, A. Molinari, M. Colone, M. Tatti, M. Sargiacomo, S. Fais, Microenvironmental pH is a key factor for exosome traffic in tumor cells, The Journal of biological chemistry, 284 (2009) 34211-34222.

[28] A.L. Arsenault, V. Ling, N. Kartner, Altered Plasma-Membrane Ultrastructure in Multidrug-Resistant Cells, Biochim Biophys Acta, 938 (1988) 315-321.

[29] L.M. Garcia-Segura, J.A. Ferragut, A.V. Ferrer-Montiel, P.V. Escriba, J.M. Gonzalez-Ros, Ultrastructural alterations in plasma membranes from drug-resistant P388 murine leukemia cells, Biochim Biophys Acta, 1029 (1990) 191-195.

[30] M. Sehested, T. Skovsgaard, B. van Deurs, H. Winther-Nielsen, Increased plasma membrane traffic in daunorubicin resistant P388 leukaemic cells. Effect of daunorubicin and verapamil, British journal of cancer, 56 (1987) 747-751.

[31] M. Sehested, T. Skovsgaard, B. van Deurs, H. Winther-Nielsen, Increase in nonspecific adsorptive endocytosis in anthracycline- and vinca alkaloid-resistant Ehrlich ascites tumor cell lines, Journal of the National Cancer Institute, 78 (1987) 171-179.

[32] L.M. Garcia-Segura, F. Soto, R. Planells-Cases, J.M. Gonzalez-Ros, J.A. Ferragut, Verapamil reverses the ultrastructural alterations in the plasma membrane induced by drug resistance, FEBS letters, 314 (1992) 404-408.

[33] W.T. Beck, The cell biology of multiple drug resistance, Biochemical pharmacology, 36 (1987) 2879-2887.

[34] R. Callaghan, L.C. van Gorkom, R.M. Epand, A comparison of membrane properties and composition between cell lines selected and transfected for multi-drug resistance, British journal of cancer, 66 (1992) 781-786.

[35] C. Federici, F. Petrucci, S. Caimi, A. Cesolini, M. Logozzi, M. Borghi, S. D'Ilio, L. Lugini, N. Violante, T. Azzarito, C. Majorani, D. Brambilla, S. Fais, Exosome release and low pH belong to a framework of resistance of human melanoma cells to cisplatin, Plos One, 9 (2014) e88193.

[36] J.L. Biedler, H. Riehm, Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic, and cytogenetic studies, Cancer research, 30 (1970) 1174-1184.

[37] C. Rauch, On the relationship between drug's size, cell membrane mechanical properties and high levels of multi drug resistance: a comparison to published data, Eur Biophys J Biophy, 38 (2009) 537-546.

[38] C.A. Doige, X. Yu, F.J. Sharom, The effects of lipids and detergents on ATPase-active P-glycoprotein, Biochim Biophys Acta, 1146 (1993) 65-72.

[39] M.M. Gottesman, S.V. Ambudkar, D. Xia, Structure of a multidrug transporter, Nat Biotechnol, 27 (2009) 546-547.

[40] Z.E. Sauna, S.V. Ambudkar, About a switch: how P-glycoprotein (ABCB1) harnesses the energy of ATP binding and hydrolysis to do mechanical work, Mol Cancer Ther, 6 (2007) 13-23.

[41] G.D. Eytan, Mechanism of multidrug resistance in relation to passive membrane permeation, Biomed Pharmacother, 59 (2005) 90-97.

[42] E. Gatlik-Landwojtowicz, P. Aanismaa, A. Seelig, The rate of P-glycoprotein activation depends on the metabolic state of the cell, Biochemistry, 43 (2004) 14840-14851.

[43] E. Landwojtowicz, P. Nervi, A. Seelig, Real-time monitoring of P-glycoprotein activation in living cells, Biochemistry, 41 (2002) 8050-8057.

[44] C. Stock, R.A. Cardone, G. Busco, H. Krahling, A. Schwab, S.J. Reshkin, Protons extruded by NHE1: digestive or glue?, Eur J Cell Biol, 87 (2008) 591-599.

446 [45] D.B. Longley, P.G. Johnston, Molecular mechanisms of drug resistance, J Pathol, 205
447 (2005) 275-292.
448 [46] C. Rauch, The "multi" of drug resistance explained by oscillating drug transporters,
449 drug-membrane physical interactions and spatial dimensionality, Cell biochemistry and
450 biophysics, 61 (2011) 103-113.
451 [47] S. Harguindey, G. Orive, J. Luis Pedraz, A. Paradiso, S.J. Reshkin, The role of pH
452 dynamics and the Na+/H+ antiporter in the etiopathogenesis and treatment of cancer.
453 Two faces of the same coin--one single nature, Biochim Biophys Acta, 1756 (2005) 1-24.
454 [48] S. Harguindey, J.L. Pedraz, R. Garcia Canero, J. Perez de Diego, E.J. Cragoe, Jr.,
455 Hydrogen ion-dependent oncogenesis and parallel new avenues to cancer prevention and
456 treatment using a H(+)-mediated unifying approach: pH-related and pH-unrelated
457 mechanisms, Crit Rev Oncog, 6 (1995) 1-33.
458 [49] R. Perona, R. Serrano, Increased pH and tumorigenicity of fibroblasts expressing a
459 yeast proton pump, Nature, 334 (1988) 438-440.
460 [50] S.J. Reshkin, A. Bellizzi, S. Caldeira, V. Albarani, I. Malanchi, M. Poignee, M. Alunni-
461 Fabbroni, V. Casavola, M. Tommasino, Na+/H+ exchanger-dependent intracellular
462 alkalinization is an early event in malignant transformation and plays an essential role in
463 the development of subsequent transformation-associated phenotypes, FASEB J, 14
464 (2000) 2185-2197.
465 [51] D. Hanahan, R.A. Weinberg, The hallmarks of cancer, Cell, 100 (2000) 57-70.
466 [52] S. Harguindey, J.L. Arranz, J.D. Polo Orozco, C. Rauch, S. Fais, R.A. Cardone, S.J.
467 Reshkin, Cariporide and other new and powerful NHE1 inhibitors as potentially selective
468 anticancer drugs--an integral molecular/biochemical/metabolic/clinical approach after one
469 hundred years of cancer research, J Transl Med, 11 (2013) 282.
470 [53] C. Daniel, C. Bell, C. Burton, S. Harguindey, S.J. Reshkin, C. Rauch, The role of proton
471 dynamics in the development and maintenance of multidrug resistance in cancer, Biochim
472 Biophys Acta, 1832 (2013) 606-617.
473 [54] S.D. Webb, J.A. Sherratt, R.G. Fish, Mathematical modelling of tumour acidity:
474 regulation of intracellular pH, J Theor Biol, 196 (1999) 237-250.
475 [55] P.D. Roepe, pH and multidrug resistance, Novartis Found Symp, 240 (2001) 232-
476 247; discussion 247-250, 265-238.
477 [56] N. Raghunand, R.J. Gillies, pH and chemotherapy, Novartis Found Symp, 240 (2001)
478 199-211; discussion 265-198.
479 [57] Q. Chen, R.S. Benson, A.D. Whetton, S.R. Brant, M. Donowitz, M.H. Montrose, C.
480 Dive, A.J. Watson, Role of acid/base homeostasis in the suppression of apoptosis in
481 haemopoietic cells by v-Abl protein tyrosine kinase, J Cell Sci, 110 (Pt 3) (1997) 379-387.
482 [58] J.F. Goossens, J.P. Henichart, L. Dassonneville, M. Facompre, C. Bailly, Relation
483 between intracellular acidification and camptothecin-induced apoptosis in leukemia cells,
484 Eur J Pharm Sci, 10 (2000) 125-131.
485 [59] C. Rauch, Toward a mechanical control of drug delivery. On the relationship between
486 Lipinski's 2nd rule and cytosolic pH changes in doxorubicin resistance levels in cancer cells:
487 a comparison to published data, European biophysics journal : EBJ, 38 (2009) 829-846.
488 [60] B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, J.D. Watson, Biologie Moleculaire
489 de La Cellule, New York, 1994.
490 [61] A.D. Petelska, Z.A. Figaszewski, Effect of pH on the interfacial tension of bilayer lipid
491 membrane formed from phosphatidylcholine or phosphatidylserine, Biochimica et
492 biophysica acta, 1561 (2002) 135-146.
493 [62] H.I. Petrache, T. Zemb, L. Belloni, V.A. Parsegian, Salt screening and specific ion
494 adsorption determine neutral-lipid membrane interactions, Proceedings of the National
495 Academy of Sciences of the United States of America, 103 (2006) 7982-7987.
496 [63] A.V. Victorov, N. Janes, T.F. Taraschi, J.B. Hoek, Packing constraints and electrostatic
497 surface potentials determine transmembrane asymmetry of phosphatidylethanol,
498 Biophysical journal, 72 (1997) 2588-2598.
499 [64] K. Sandvig, S. Olsnes, O.W. Petersen, B. van Deurs, Acidification of the cytosol
500 inhibits endocytosis from coated pits, The Journal of cell biology, 105 (1987) 679-689.
501 [65] F. Luciani, M. Spada, A. De Milito, A. Molinari, L. Rivoltini, A. Montinaro, M. Marra, L.
502 Lugini, M. Logozzi, F. Lozupone, C. Federici, E. Iessi, G. Parmiani, G. Arancia, F. Belardelli,

S. Fais, Effect of proton pump inhibitor pretreatment on resistance of solid tumors to cytotoxic drugs, Journal of the National Cancer Institute, 96 (2004) 1702-1713.

[66] S. Penman, Rethinking cell structure, Proceedings of the National Academy of Sciences of the United States of America, 92 (1995) 5251-5257.

[67] S. Fais, Moulding the shape of a metastatic cell, Leukemia research, 34 (2010) 843-847.

[68] F. Luciani, A. Molinari, F. Lozupone, A. Calcabrini, L. Lugini, A. Stringaro, P. Puddu, G. Arancia, M. Cianfriglia, S. Fais, P-glycoprotein-actin association through ERM family proteins: a role in P-glycoprotein function in human cells of lymphoid origin, Blood, 99 (2002) 641-648.

[69] D. Brambilla, S. Zamboni, C. Federici, L. Lugini, F. Lozupone, A. De Milito, S. Cecchetti, M. Cianfriglia, S. Fais, P-glycoprotein binds to ezrin at amino acid residues 149-242 in the FERM domain and plays a key role in the multidrug resistance of human osteosarcoma, International journal of cancer, 130 (2012) 2824-2834.

[70] S. Fais, M.O. Fauvarque, TM9 and cannibalism: how to learn more about cancer by studying amoebae and invertebrates, Trends in molecular medicine, 18 (2012) 4-5.

[71] M. Walsh, S. Fais, E.P. Spugnini, S. Harguindey, T. Abu Izneid, L. Scacco, P. Williams, C. Allegrucci, C. Rauch, Z. Omran, Proton pump inhibitors for the treatment of cancer in companion animals, Journal of experimental & clinical cancer research : CR, 34 (2015) 93.

[72] E.P. Spugnini, T. Azzarito, S. Fais, M. Fanciulli, A. Baldi, Electrochemotherapy as First Line Cancer Treatment: Experiences from Veterinary Medicine in Developing Novel Protocols, Current cancer drug targets, 16 (2016) 43-52.

[73] E.P. Spugnini, S. Buglioni, F. Carocci, M. Francesco, B. Vincenzi, M. Fanciulli, S. Fais, High dose lansoprazole combined with metronomic chemotherapy: a phase I/II study in companion animals with spontaneously occurring tumors, J Transl Med, 12 (2014) 225.

[74] E.P. Spugnini, A. Baldi, S. Buglioni, F. Carocci, G.M. de Bazzichini, G. Betti, I. Pantaleo, F. Menicagli, G. Citro, S. Fais, Lansoprazole as a rescue agent in chemoresistant tumors: a phase I/II study in companion animals with spontaneously occurring tumors, J Transl Med, 9 (2011) 221.

[75] S. Ferrari, F. Perut, F. Fagioli, A. Brach Del Prever, C. Meazza, A. Parafioriti, P. Picci, M. Gambarotti, S. Avnet, N. Baldini, S. Fais, Proton pump inhibitor chemosensitization in human osteosarcoma: from the bench to the patients' bed, J Transl Med, 11 (2013) 268.

[76] B.Y. Wang, J. Zhang, J.L. Wang, S. Sun, Z.H. Wang, L.P. Wang, Q.L. Zhang, F.F. Lv, E.Y. Cao, Z.M. Shao, S. Fais, X.C. Hu, Erratum to: Intermittent high dose proton pump inhibitor enhances the antitumor effects of chemotherapy in metastatic breast cancer, Journal of experimental & clinical cancer research : CR, 34 (2015) 109.

[77] B.Y. Wang, J. Zhang, J.L. Wang, S. Sun, Z.H. Wang, L.P. Wang, Q.L. Zhang, F.F. Lv, E.Y. Cao, Z.M. Shao, S. Fais, X.C. Hu, Intermittent high dose proton pump inhibitor enhances the antitumor effects of chemotherapy in metastatic breast cancer, Journal of experimental & clinical cancer research : CR, 34 (2015) 85.

[78] R. Falcone, M. Roberto, C. D'Antonio, A. Romiti, A. Milano, C.E. Onesti, P. Marchetti, S. Fais, High-doses of proton pump inhibitors in refractory gastro-intestinal cancer: A case series and the state of art, Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver, 48 (2016) 1503-1505.

[79] R.C. Friedberg, C.A. Rauch, The role of the medical laboratory director, Clin Lab Med, 27 (2007) 719-731, v.

553   Legends

554

555   Figure 1: (A) Lipid asymmetry at the vesicular scale: Given the small size of vesicles, the radius

556   and membrane thickness are relatively close together ($R/h \sim 10$). Thus, the outer leaflet of a

557   vesicle (S_{out}) has significantly more lipids than the inner leaflet (S_{in}). As the vesicle is

558   spherical, noting $S_0 = 4\pi R^2$ the neutral surface area namely the surface area between the outer

559   and inner leaflets, it follows at the first order that $S_{out} = 4\pi(R+h/2)^2 \sim S_0(1+h/R)$ and

560    $S_{in} = 4\pi(R-h/2)^2 \sim S_0(1-h/R)$. Thus $S_{out} - S_{in} \sim S_0 \cdot h/R$. (B) Sketch representing the

561   current model linking fluid phase endocytosis to the membrane phospholipid number

562   asymmetry [14]. In the left panel, the translocation of dark-headed lipids into the inner leaflet

563   induces a differential packing of lipids between leaflets leading to membrane bending and

564   vesiculation [13, 15]. Note the membrane recycling that occurs in cells (right panel), i.e. the

565   exocytosis of vesicles with a size similar to endocytic vesicles, allows the maintenance of lipid

566   asymmetry and thus the maintenance of the differential packing of leaflets at the level of the

567   plasmalemma. Accordingly, the lipid number asymmetry has been experimentally deduced

568   from studies on drug sensitive cells (K562) with a value $\Delta N/N_0 = 4\%$ providing a ~35nm

569   vesicle radius [13]. (C) Representation of the different energy barriers (noted together $U(x)$)

570   and involved when a drug traverses the bilayer cellular membrane. Two leaflets have been

571   represented with an inner leaflet containing more phospholipids related to the increase in the

572   difference in surface tensions (upper graph). Energy profiles of lipid packing in both leaflet

573   (plain curve-middle graph) and hydrophobic core of membrane (dashed curve-middle graph)

574   are both involved in providing penalty energies with regard to the transbilayer movement of

575   drugs. As the inner leaflet is packed, drugs crossing the membrane will be trapped in this leaflet

576   which will delay and impair their flow into the cytosol [79]. The latter effect will be dependent
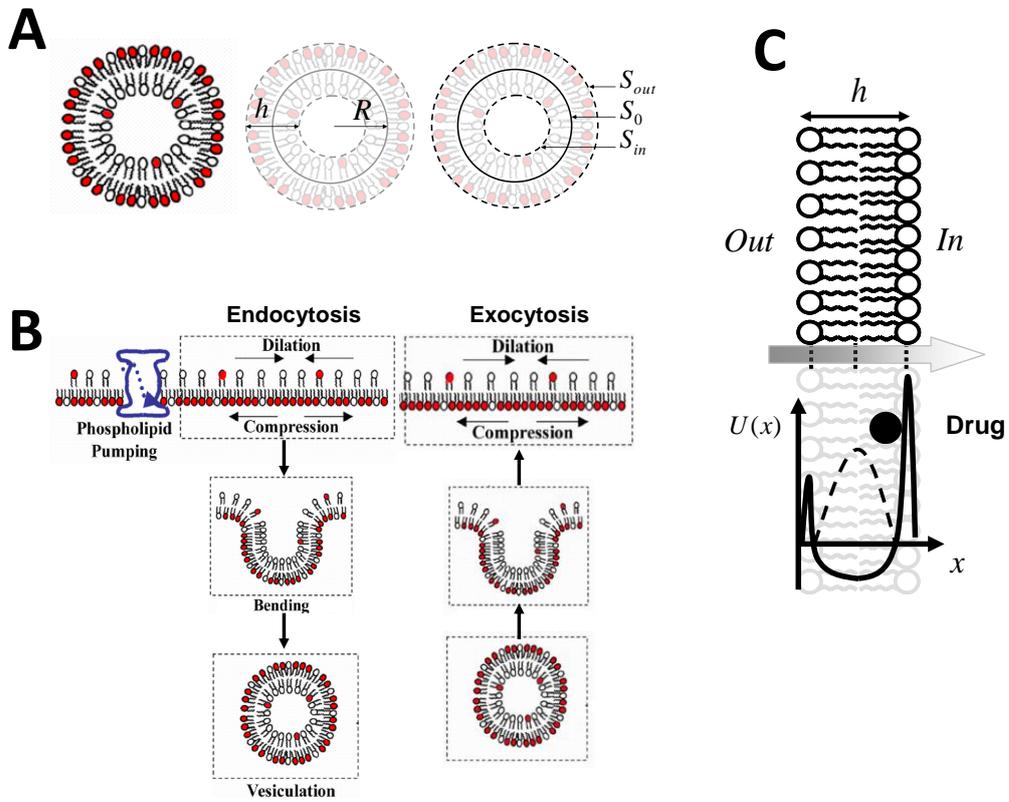
577   on the size of drugs as bigger drugs will "feel more strongly" this mechanical barrier. In the

578   present paper, this effect is supposed to be central for the high levels of cross resistance to

579   drugs.

580

581   Figure 2: (A) Comparison between experimentally measured doxorubicin resistance levels

582   obtained in cells (blanked circles) and the theory (filled circles). The open circles

583   corresponding to SW1573 (lung derived cancer cells), K562R (leukemic cancer cells) and

584   MCF-7R (breast derived cancer cells) are indicated with arrows and labels. Finally the straight

585   line is the linear regression of experimental data which agrees very well with the theory.

586   Figure 1

587

A

S_{out}
S_0
S_{in}

h   *R*

B

Endocytosis

Dilation

Compression

Phospholipid
Pumping

Bending

Vesiculation

Exocytosis
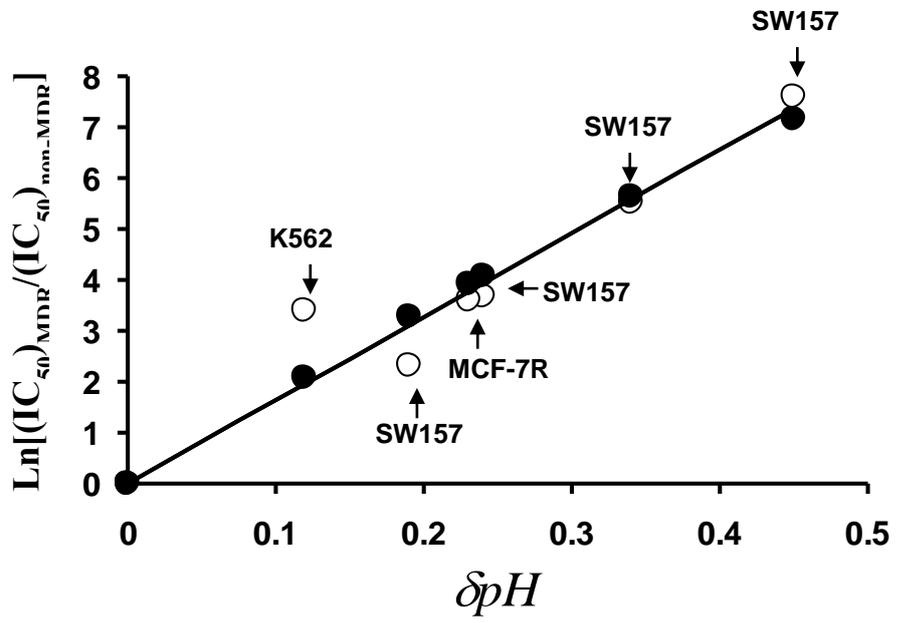
Dilation

Compression

C

h

Out   *In*

$U(x)$ **Drug**

x

588

589

590   Figure 2

591

592