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Re-evaluation of the mechanism of cytotoxicity of dialkylated lariat ether compounds†

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The cytotoxicity of dialkylated lariat ethers has been previously attributed to their ionophoric properties. Herein, we provide evidence that these effects are due to loss of membrane integrity rather than ion transport, a finding with important implications for the future design of synthetic ionophores.

Since the first report of their synthesis,[1,2] crown ethers and their derivatives have generated wide interest due to their ability to form stable complexes with cations.[2] These properties have been successfully exploited in ion transport through bulk liquid membranes[3,4] as well as in sensors and scaffolds for materials,[5] developments which prompted their examination as biologically relevant ionophores.[6–8] The transport of ions through biological membranes underlies many key physiological processes[9–18] and understanding the complexities of this phenomenon continues to be an area of active research. Crown ethers are potentially powerful tools in this pursuit, due to their binding properties and highly customizable structures. Indeed, crown ether derivatives, such as monoalkylated[19] and dialkylated lariat ethers,[20,21] amphiphilic benzo(crown) ether derivatives,[22] hydraphiles,[23] and ion shuttles[24] have been demonstrated to function as ionophores. Despite these successful examples, our understanding of the mechanisms of ion transport by these crown ether derivatives remains extremely limited. An improved understanding of these processes will provide critical insights that will both advance fundamental knowledge about ion transport mechanisms and provide a framework for the rational design of synthetic ionophores with well-defined properties.

Towards this aim, we identified previously reported dialkylated diaza crown ethers[20,21] as an ideal starting point. Lariat ethers are crown ether analogues with one or more sidearms attached to the macrocyclic core structure.[25] The possibility of adding customized pendant groups to the crown ether core allows a high degree of selective modification to their physical parameters.[25] Lariat ethers have also been reported to bind alkali cations and behave as ionophores in bulk liquid membranes and ion-selective electrodes.[26–29] The dialkylated diaza(18-crown-6) ethers, a subset of the lariat class, have been reported to have toxic activity towards prokaryotic and eukaryotic cells.[20] Evidence from toxicity and depolarization assays initially suggested that these compounds behave as ion carriers.[20] However, experiments in asolectin bilayers revealed that dioctylated and diundecylated lariat ethers elicit discrete increases in membrane conductance,[21] a result typical of ion channels, as opposed to ion carriers. Moreover, the effect of the alkyl chain lengths on the toxicity and transport implied that the interaction of these compounds with a bilayer membrane differs from their behaviour in a bulk liquid membrane. In general, the literature suggests that hydrophobic lariat ethers that bear longer alkyl chains function as more efficient cation carriers.[20,30] In contrast, dialkylated lariat ethers show peak activity when a 10 carbon chain is present on the core, with the activity diminishing with increasing chain length.[20] This observation prompted Leevy and co-workers to propose that dialkylated lariat ethers require a minimum hydrophobicity to act as ion carriers, but when the alkyl chains are too long, the molecules are able to nest inertly within the membrane.[20] In order to test this mechanism and establish a deeper understanding of their transport behavior in membranes, we focused on a representative set of lariat ethers bearing dialkylated tails ranging from 6 to 14 carbons.

The series of dialkylated diaza(18-crown-6) ether were prepared by a simple one-step reductive amination of 4,13-diaza(18-crown-6) and the appropriate aldehyde (Fig. 1; Methods in ESI†), as opposed to previous two-step procedures.[20,21] The notation LEC_n refers to the dialkylated lariat ether substituted with an alkyl chains of n carbons. The cytotoxicity was determined by measuring the minimum inhibitory concentrations (MIC) for this series of dialkylated lariat ethers in the Gram-negative bacteria *Escherichia coli*, the Gram-positive bacteria *Bacillus subtilis*, and human embryonic kidney (HEK293T) cells. The results showed that *B. subtilis* is more susceptible to LEC_6–LEC_{14}, as judged by lower MICs; LEC_{10} is the most toxic to *E. coli* (MIC = 10 μM), while LEC_{10}, LEC_{11}, and LEC_{12} are the most toxic to *B. subtilis* at concentrations as low as 2 μM (Fig. S1†), results that are in good agreement with similar

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† Electronic supplementary information (ESI) available: Methods, NMR and MS data, MIC determination, $DiSC_3(5)$ release assays. See DOI: 10.1039/d0ra08494h

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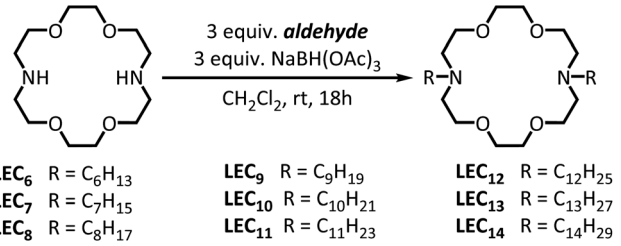

Fig. 1 Synthesis of dialkylated diaza(18-crown-6) ethers compounds.

studies[20] (Fig. S1, ESI†). We also confirmed a remarkable discontinuity in the toxicity between LEC_{12} and LEC_{13}, where the addition of only a single methylene group to the alkyl chain completely abrogates the toxicity towards *B. subtilis* from a MIC = 2 μM with LEC_{12} to undetectable with LEC_{13} at concentration as high as 400 μM. The toxicities of dialkylated lariat ethers towards HEK293T cells were more consistent than the trends with *E. coli* and *B. subtilis*; however, LEC_{10} (MIC = 6 μM) proved to be the most toxic lariat ether towards all three tested cellular systems.

Dialkylated lariat ethers LEC_6–LEC_{14} were then tested for their ability to depolarize a *B. subtilis* membrane using the fluorescent dye 3,3-dipropylthiadicarbocyanine ($DiSC_3(5)$), which undergoes membrane voltage-dependent partitioning between the intracellular and the extracellular medium.[31] Cell hyperpolarization (more negatively charged inside the cell) results in an uptake of the dye, while cell membrane depolarization (more positively charged inside the cell) results in a release of the dye. The accumulation of the dye in the interior of the cell can be detected by a decrease in fluorescence due to self-quenching,[31] which enables the dye to be utilized as an indirect reporter of changes in cell membrane voltage.[32] As the resting membrane voltage in *B. subtilis* is approximately −120 mV,[33] $DiSC_3(5)$ quickly accumulates inside intact bacteria (timepoint t_2 in Fig. S2a–i†). The addition of up to 60 mM KCl (violet curve, timepoint t_3 in Fig. S2a–i†) does not cause substantial membrane depolarization, as determined by the limited increase in fluorescence, mainly because the endogenous K^+ transporters and channel activities[34–38] are inhibited at 25 °C. However, upon addition of the dialkylated lariat ether (timepoint t_4 in Fig. S2a–i†), ions move down the electrochemical gradient and give rise to membrane depolarization, as evidenced by an increase in fluorescence. The effects of the alkyl chain length on the relative $DiSC_3(5)$ release after 10 minutes following addition of the dialkylated lariat ether (Fig. S3†) were qualitatively similar to those reported previously.[20] The dialkylated lariat ethers with the highest toxicities elicited faster $DiSC_3(5)$ efflux in the presence of K^+, suggesting they are more efficient at transporting cations (Fig. S2a–i and S3; † violet curves). As the induction of membrane depolarization is consistent with an ionophoric mechanism, the next step was to determine the cation selectivity exhibited by this class of the compounds.

Binding to ionophores requires at least a partial substitution of water molecules in the hydration sphere by ions to achieve an ionophore-like transport mechanism. Thus, some degree of ion selectivity is expected,[26–29] as observed in the case of valinomycin, a natural carrier-type ionophore that is extremely selective for K^+ over Na^+ [39,40] and does not transport *N*-methyl-D-glucamine ($NMDG^+$). When depolarization assays were performed in the presence of different cations, valinomycin promoted $DiSC_3(5)$ release only when K^+ was added to the external solution, but not when Na^+ or $NMDG^+$ was added (Fig. 2a and b). Conversely, when LEC_{10} was tested under the same conditions, no significant differences in relative $DiSC_3(5)$ release were observed (Fig. 2c and d). Furthermore, our control experiments show that even in the absence of the alkali cation (Fig. S2a–i, and S3;† black curves), the relative $DiSC_3(5)$ release rates were similar to those observed in the presence of K^+ (Fig. S2a–i and S3;† violet curve). This unexpected result forced us to consider two possible explanations. Either the dialkylated lariat ethers behave as non-selective ionophores that are capable of transporting large cations, such as $NMDG^+$, or their primary effect is to disrupt membrane integrity, *i.e.* the $DiSC_3(5)$ efflux is due to the lysis of the cells, rather than ion transport across the membrane.

To determine whether transport of $NMDG^+$ can account for the observed efflux of $DiSC_3(5)$ from cells, we tested LEC_{10} activity in a cation-free dextrose solution (see Methods, ESI†). In this experiment, the only cation in the external solution (if any)

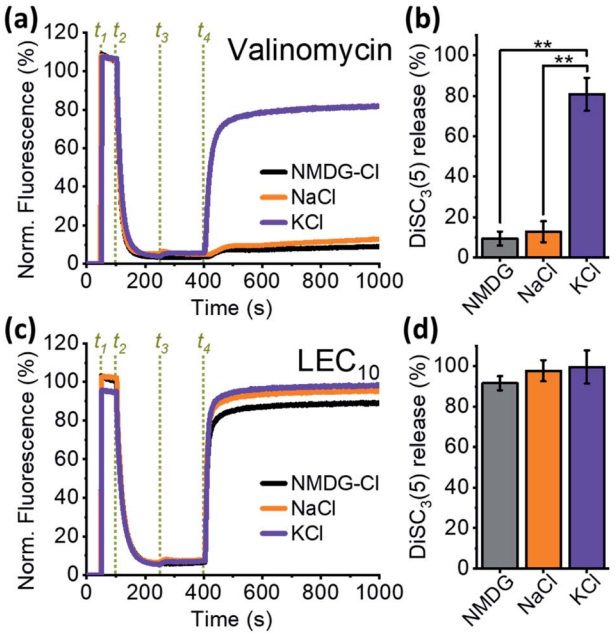

Fig. 2 Comparison between the selectivity of valinomycin and LEC_{10}: (a) normalized changes in $DiSC_3(5)$ fluorescence due to the activity of 2 μM valinomycin; events in green are dye additions (t_1), *B. subtilis* cells (t_2), concentrated salts up to 60 mM KCl (violet), NaCl (orange) or 60 mM NMDG-Cl (black) (t_3), and valinomycin up to 2 μM (t_4); (b) fluorescence values at the end of the experiment, as shown in panel a (average ± S.E.M); (c) normalized changes in $DiSC_3(5)$ fluorescence due to the activity of 2 μM LEC_{10}; the experiment is identical to that in panel a, except LEC_{10} was added in t_4; (d) fluorescence at the end of the experiment as shown in panel c (average ± S.E.M). A minimum of three experiments for each condition were averaged in panels b and d.

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is added at timepoint t_3 (Fig. S4a†). Addition of 2 μM LEC_{10} produced a large $DiSC_3(5)$ release in the presence of KCl (Fig.-S4a,† violet curve), but interestingly, this same effect was also observed in the absence of any external cation (Fig. S4a and b,† black curve; additional dextrose solution was added at timepoint t_4). The independence of the $DiSC_3(5)$ efflux from the identity or the presence of the external cations is not compatible with an ionophore-like mechanism.

Previous studies indicated that lariat ethers reported to display the ability to collapse membrane potential in depolarization assays also exhibited ion channel-like activity in bilayers. This suggested that we might employ similar depolarization assays to serve as a convenient surrogate for measuring electrical activity. We tested our most potent compound, LEC_{10}, for ion channel activity in planar lipid bilayers. As a control, unitary channels of the ionophore gramicidin were recorded in the presence of a KCl solution (150 mM) (Fig. S5a†). However, no ion channel activity was detected in a similar experiment performed using 2 μM LEC_{10} (Fig. S5b†), even after one hour of recording. Variations in the LEC_{10} concentration (10, 100, and 200 μM), as well as the KCl concentration (up to 500 mM), including replacing KCl with NaCl (see Methods, ESI†), resulted in no indication of ion channel activity. This lack of unitary channel formation, or even a carrier-like increase of the conductance, precludes an ionophore-like mechanism of ion transport.

Given our findings that this class of dialkylated lariat ether derivatives do not act as typical ionophores, we next considered the possibility that the activity of these compounds results from disruption of membrane integrity. A well-established assay was utilized to measure cell lysis by monitoring the release of the enzyme lactate dehydrogenase (LDH), which is ubiquitous in the cytoplasm of all cell types. The tetrameric active form of LDH catalyzes the final step of the glycolysis in *B. subtilis* and has a molecular weight of approximately 146 kDa in,[41,42] with a nearly globular shape of an approximated radius of 80 Å (PDB ID: 3PQD). The release of proteins of this size demonstrates the test compounds are likely to cause cell lysis but we cannot rule out the possibility that they form large pores. LDH couples two redox reactions, the first involving the interconversion of pyruvate (oxidized) and L-lactate (reduced) and the second, the interconversion of NAD^+ (oxidized) and NADH (reduced). The NADH oxidation can be coupled to the diaphorase-catalyzed oxidation of resazurin to resofurin, which is highly fluorescent (Fig. 3a). When the concentrations of L-lactate, NAD^+, diaphorase, and resazurin are saturated, the rate of increase in the resofurin fluorescence is limited only by the amount of available LDH in the medium. Our experiments show that the addition of LEC_{10} to a mixture containing *B. subtilis* cells and the other components necessary for the LDH assay (see Methods, ESI†) causes a rapid rise in the fluorescence (Fig. 3b and c), indicating loss of membrane integrity. In contrast, the addition of the same amount of the trifluoroethanol (TFE) solvent used to dissolve LEC_{10} did not produce any increase in the fluorescence (Fig. 3b and c).

In summary, we report one-step syntheses of dialkylated diaza(18-crown-6) ether derivatives and an in-depth evaluation

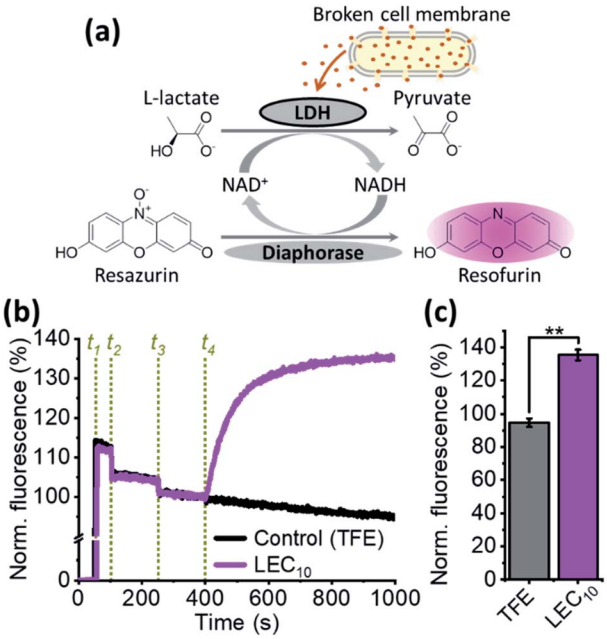

Fig. 3 Lactate dehydrogenase release from *B. subtilis* in response to LEC_{10} treatment: (a) two coupled redox reactions lead to increased fluorescent resofurin concentration when lactate dehydrogenase (LDH) is released from lysed cells; (b) normalized time course of the resofurin fluorescence; events in green are additions of resazurin (t_1); *B. subtilis* cells (t_2), diaphorase (t_3), and up to 2 μM LEC_{10} (magenta curve) or the same volume of TFE (control, black curve) (t_4); (c) normalized resofurin fluorescence at the end of the experiment as shown in panel b (average ± S.E.M).

of their behaviour as ionophores for cell membranes. The acute release of LDH, a complete lack of ion specificity, depolarization in the absence of extracellular ions, and a lack of discrete changes in the conductance in the planar lipid bilayers solidly demonstrates that biological activities of these lariat ethers are due to their membrane lytic activity, as opposed to the expected ion transport activity. Our findings warrant re-evaluation of the mechanisms of activity of many previously reported synthetic ionophores, which have been classified as such based only on cell survival assays and depolarization assays, without attention to detailed electrical activity measurements. We also note that even in cases where ionophores do not show electrical activity, due to transport *via* carrier type mechanisms, our control experiments are able to discriminate between ion carrier and lytic activity.

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Conflicts of interest

There are no conflicts to declare.

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