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Systematically altering the lipophilicity of rhenium(I) tricarbonyl anticancer agents to tune the rate at which they induce cell death†

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Rhenium-based anticancer agents have arisen as promising alternatives to conventional platinum-based drugs. Based on previous studies demonstrating how increasing lipophilicity improves drug uptake within the cell, we sought to investigate the effects of lipophilicity on the anticancer activity of a series of six rhenium(I) tricarbonyl complexes. These six rhenium(I) tricarbonyl structures, called Re-Chains, bear pyridyl imine ligands with different alkyl chains ranging in length from two to twelve carbons. The cytotoxicities of these compounds were measured in HeLa cells. At long timepoints (48 h), all compounds are equally cytotoxic. At shorter time points, however, the compounds with longer alkyl chains are significantly more active than those with smaller chains. Cellular uptake studies of these compounds show that they are taken up *via* both passive and active pathways. Collectively, these studies show how lipophilicity affects the rate at which these Re compounds induce their biological activities.

Developing new drugs is an iterative process and requires optimization of lead candidates to improve their biological efficacies. Several factors contribute to the success of potential drug candidates and are addressed during this optimization process. These characteristics include good solubility, stability, permeability, drug absorption, and pharmacokinetics.[1–5] The lipophilicity of a compound, often measured as an octanol–water partition coefficient (log *P*) value, can have large effects on all of these properties and is, therefore, often modified systematically during these efforts.[6–13] For example, Lipinski's rule of 5, an empirical set of guidelines for identifying molecules with "drug-like" properties, requires that drug candidates possess log *P* values of less than five.[14–16] The basis for this rule is likely a consequence of the fact that log *P* values affect the cellular uptake, cytotoxic potency, and protein-binding of drug candidates.[1,6,7,17–19] Log *P* values that exceed five may potentially lead to increased activity and enhanced liver and lung uptake, resulting in diminished selectivity and off-target side effects.[20]

Primarily motivated by the success of cisplatin and related platinum-containing drugs, a large number of efforts in recent years focused on developing new metal-based anticancer agents.[21] Similar to conventional organic drug candidates, these metal-containing compounds have biological activities that are modulated by their relative lipophilicities. The introduction of variable-size alkyl chains in metal complexes to systematically alter their lipophilicities, for example, has given rise to promising complexes of platinum[22–25] for anticancer therapy. Complexes of the third-row transition metal, rhenium, and its radioactive congener, technetium-99m (^{99m}Tc), have also been studied in this context, and in some cases their lipophilicities have been correlated to their cytotoxic activities.[26–28] Collectively, these studies highlight how transition metal compounds, like conventional organic drug candidates, can be modified to tune their biological properties.

Based on our group's prior investigations on the anticancer potential of rhenium(I) tricarbonyl ($Re(CO)_3$) complexes,[29–34] we sought to evaluate how systematically altering the lipophilicity of this class of compounds affects biological activity. To explore this hypothesis, a series of $Re(CO)_3$ complexes bearing pyridyl imine Schiff-base ligands with pendent alkyl chains ranging from two to twelve carbons was prepared. Our evaluation of their cytotoxic activities and cellular uptake in HeLa cells revealed that the more lipophilic compounds were able to trigger cell death more rapidly than their hydrophilic analogues. This study provides an unusual direct example of how compound lipophilicity can affect the rate of cancer cell death induction and highlights how time-dependent measurements may give valuable insight on the investigation of new drug candidates.

Our efforts to prepare a series of $Re(CO)_3$ compounds with varying linear carbon chain lengths were motivated by a

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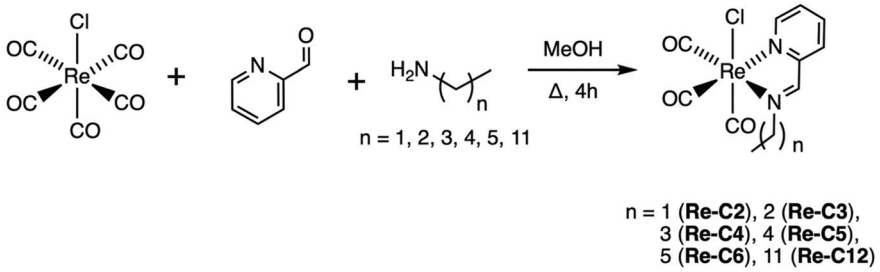

Scheme 1 General synthetic approach and structures of **Re-Chains** complexes.

related previous study.[27] In this prior study, $Re(CO)_3$ complexes bearing axial alkylimidazole ligands were investigated in different biological models. Notably, it was observed that compounds with longer alkyl chains have increased cytotoxic activity in the anaerobically grown aerotolerant protistan fish parasite, *spiro nucleus vortens*, cells. To build upon these prior efforts, we sought to test the role of having long carbon chains on the equatorial diimine, rather than the axial, ligands of these complexes.

To prepare this class of compounds, we used highly modular chemistry, largely developed by the group of Ziegler,[35–44] to prepare $Re(CO)_3$ complexes bearing pyridyl imine ligands with variable length alkyl chains. Following this approach, we mixed $Re(CO)_5Cl$, picolinaldehyde, and variable chain length alkyl amines in refluxing methanol to afford the compounds shown in Scheme 1, collectively referred to as **Re-Chains**. These six complexes generally abide by Lipinski's rule of 5, which dictates that drug-like molecules have less than five hydrogen bond donors, less than ten hydrogen bond acceptors, less than 500 Da in molecular weight, and a log *P* value less than five.[14–16] **Re–C12** has a molecular weight exceeding 500 Da, but we note that this aspect of Lipinski's rule most likely does not strictly apply to inorganic complexes for which certain metal atoms carry a significantly large portion of the whole molecular mass.

Following the one-pot syntheses of these six complexes, they were characterized by ^{1}H NMR spectroscopy (Fig. S1–S6, ESI†), Fourier Transform infrared (FTIR) spectroscopy (Fig. S7–S12, ESI†), UV–vis spectroscopy (Fig. S13, ESI†), electrospray ionization mass spectrometry (ESI-MS) (Fig. S14–19, ESI†), and elemental analysis (EA). The ^{1}H NMR spectra display a diagnostic imine proton that resonates at 9.23–9.24 ppm, marking an upfield shift from the parent aldehyde at 9.98 ppm. The FTIR spectra reveal three intense C≡O stretching modes, consistent with complexes of C_1 symmetry, in which the two low-energy modes range in energy from 1880 to 1930 cm^{-1} and the high-energy modes range from 2019 to 2027 cm^{-1}. The UV–vis spectral data for the complexes in acetonitrile (MeCN) reveal two prominent electronic transitions: a high-energy peak at 290 nm assigned to the intraligand π–π* transition and a lower-energy peak at 430 nm assigned to a metal-to-ligand charge transfer (MLCT) transition. The compounds were also characterized by ESI-MS, which predominantly displayed an *m*/*z* peak corresponding to the $[M - Cl]^+$ ion. Finally, the log *P*

Table 1 Log *P* values of **Re-Chains** and their free ligands

	log P^a		Calculated log P^b
Re–C2	1.59	**C2**	1.45
Re–C3	2.16	**C3**	1.90
Re–C4	2.44	**C4**	2.58
Re–C5	2.80	**C5**	3.14
Re–C6	2.91	**C6**	3.68
Re–C12	2.95	**C12**	6.79

[a] Determined using the shake-flask method after 30 min of mixing octanol and water. [b] Calculated using the ALOGPS 2.1 software.

values of the $Re(CO)_3$ complexes were determined as water–octanol partition coefficients using the shake-flask method,[45] and the log *P* values were calculated for the free equatorial ligands using the ALOGPS 2.1 program (Table 1).[46,47] As expected, both the complex, **Re–C12**, and its free ligand, **C12**, are the most lipophilic compounds (log *P* = 2.95 and 6.79, respectively), whereas **Re–C2** and **C2** are the least lipophilic (log *P* = 1.59 and 1.45, respectively). We note that the experimentally measured log *P* values for our **Re-Chains** do not differ as greatly as the calculated values for the free ligands. We hypothesize that this discrepancy may arise from time-dependent aquation and hydrolysis processes at the Re centers, which will alter the measured lipophilicity values. Despite the small differences for the **Re-Chains**, these values demonstrate the increase in lipophilic character of the compounds as a consequence of incorporating longer alkyl chains.

Having synthesized and fully characterized **Re-Chains**, we sought to evaluate their *in vitro* anticancer activities *via* dose-escalation studies in HeLa cervical cancer cells. When HeLa cells were treated with these compounds for a 48 h incubation period, all rhenium complexes exhibited 50% growth inhibitory concentration (IC_{50}) values of approximately 15 μM (Fig. 1a), compared to cisplatin which has an IC_{50} value of 9.8 μM in the same cell line.[34] This result, showing all six structures to possess equivalent cytotoxic activity, appeared to contrast our hypothesis regarding the role of lipophilicity in mediating the biological properties of this compound class. We reasoned, however, that the lack of differences in cytotoxic activities between these substantially different lipophilic complexes may lie in the rate at which they induce their cytotoxic effects. To test this hypothesis, we treated cells with the **Re-Chains** (50 μM) for varying incubation times, allowing recovery

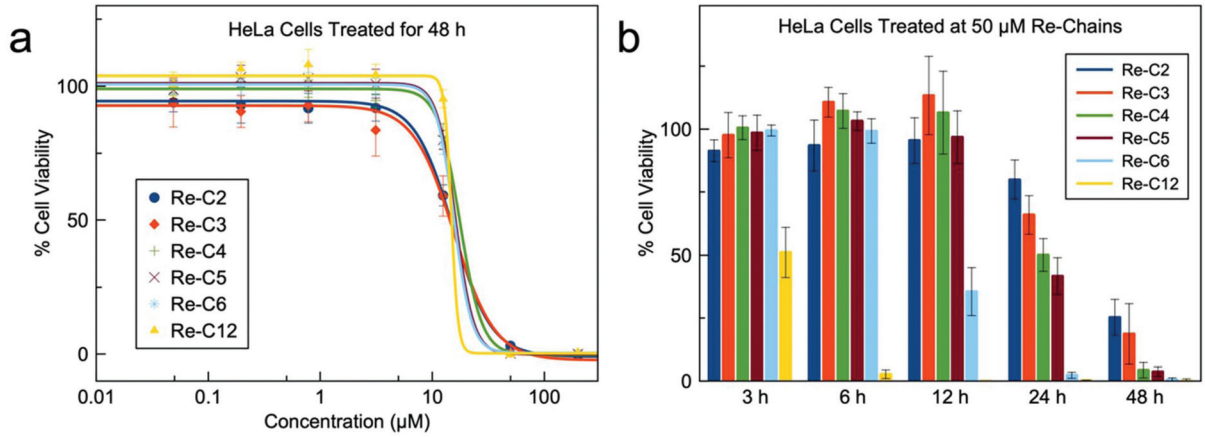

Fig. 1 (a) Dose–response curves and (b) time-dependent cell viability studies of HeLa cells treated with **Re–C2** (navy blue), **Re–C3** (red), **Re–C4** (green), **Re–C5** (maroon), **Re–C6** (light blue), **Re–C12** (yellow). The error bars represent the standard deviation from six replicates.

time to keep the duration of the assay at 48 h (Fig. 1b). Our results indicate that there is a time-dependence of the cytotoxic activity of these compounds that depends on the alkyl chain length. Notably, more lipophilic compounds with long alkyl chains, like **Re–C12**, induce their cytotoxic effects on a much faster time scale than the less lipophilic analogues. For example, treatment for 6 h with 50 μM **Re–C12** kills >95% of the cells, whereas the other five compounds have no effect. By 48 h, all six compounds decrease cell viability below 30%, consistent with the similar IC_{50} values that we measured at this time point. Thus, these results indicate that lipophilicity does play a role in mediating the cytotoxic activities of these compounds; however, this effect is not readily observed at longer time points. Presumably, long incubation times allow the less lipophilic compounds to accumulate in the cells at equipotent concentrations as the more lipophilic species.

We next measured the cellular uptake of these compounds to explore the role of lipophilicity. HeLa cells were treated with 50 μM **Re-Chains** at both 37 °C and 4 °C for 3 h, after which the cells were harvested, digested and analyzed for Re content *via* inductively coupled plasma optical emission spectroscopy (ICP-OES). The low-temperature (4 °C) incubation was used as a means of shutting down active, or energy-dependent, transport pathways through the cell membrane. The measured cellular uptake of the **Re-Chains** compounds (at both temperatures) is shown in Fig. 2. It is apparent from these data that cellular uptake scales proportionally with both the length of the carbon chain of the complex and the ligand log *P* values. The differences in cellular uptake are consistent with the different cytotoxic effects that we see in Fig. 1 for the 3 h time point, confirming that lipophilicity plays a mutually important role in uptake and cytotoxicity. Additionally, cell uptake at 4 °C is notably less than that for 37 °C. These findings suggest that the **Re-Chains** compounds are taken up, at least in part, by active transport. For related metal-based anticancer agents, like $[(\eta^6\text{-}p\text{-cymene})Os^{II}(N,N\text{-dimethylphenylazopyridine})X]^+$ in which X = Cl or I,[48] cellular uptake at 4 °C was diminished by factors of 20–30. In the case of **Re-Chains**, however, we only

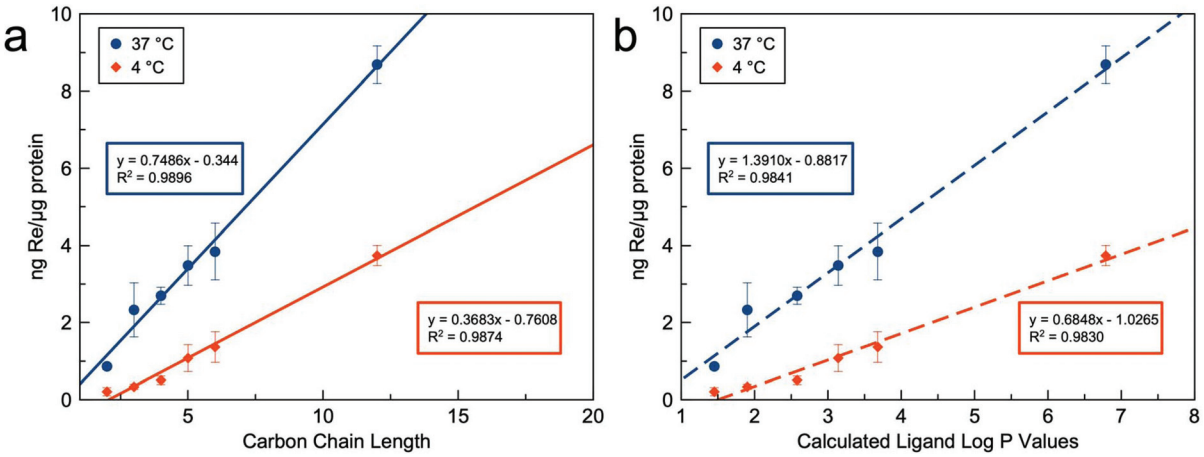

Fig. 2 Cellular uptake of **Re-Chains** after incubating for 3 h at 37 °C (blue) and 4 °C (red) in relation to (a) carbon chain length and (b) calculated log *P* values for the free ligands. The error bars represent the standard deviation from three replicates.

observe differences ranging from 2.3–7-fold (Table S1, ESI†). We interpret that moderate decreases in uptake of **Re-Chains** upon incubation at 4 °C, in comparison to related actively transported metal-based anticancer agents, reflects how passive transport is their dominant mechanism of uptake. As an alternative explanation, lower uptake could be due to precipitation of this compound at this lower temperature. However, no visible precipitation was observed during these low-temperature experiments, leading us to disfavor this hypothesis. Furthermore, even at 4 °C, the cellular uptake of **Re-Chains** still scales linearly with the carbon chain length and calculated ligand log *P* values, suggesting that lipophilicity is important for cellular uptake under both conditions.

In summary, we have prepared a small set of $Re(CO)_3$-diimine complexes bearing varying alkyl chain lengths using a three-component, one-pot reaction. In studying their cytotoxic effects and cellular uptake in HeLa cells, it was found that the more lipophilic compounds induce *in vitro* anticancer activities at much shorter time points. This result is most likely a consequence of faster cellular uptake kinetics for more lipophilic compounds. Although it has been more commonly noted that lipophilicity of drug candidates affects their biological activity, few studies to date, including a recent report involving $Re(CO)_3$ complexes,[49,50] have shown that many of these effects exhibit a time dependence. This observed time dependence on uptake and cytotoxicity, for example, could have important effects in the field of ^{99m}Tc-based radiopharmaceutical agents, for which their short half-lives require that cellular uptake and localization proceed rapidly.

Conflicts of interest

The authors declare no competing financial interests.

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