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Polymeric Organoiron Compounds as Prodrugs in Cancer Research*

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SUMMARY: Ferrocene, di(η^5-cyclopentadienyl)iron(II), has for nearly half a century now been a focal point of research activities in the realm of organo-transition-metal chemistry and physics, with ramifications into numerous technologies. More recent years have witnessed the emergence of a new research trend, probing the behavior of ferrocene in the biological realms, notably in the transformed, *i.e.* cancerous, cell system. Following initial reports attesting to the pronounced antiproliferative properties of certain water-soluble derivatives of ferrocene and its one-electron oxidation product, the ferricenium radical cation, earlier programs were set up in the author's laboratory with the objective of developing water-soluble polymeric conjugates in which the bioactive ferrocene unit is bioreversibly tied to macromolecular carriers in order to enhance its therapeutic effectiveness. In this article, these earlier investigations of polymer-ferrocene conjugation are briefly reviewed, and the current, considerably broadened synthetic program is introduced. The carriers are predominantly of the highly versatile poly(aspartamide) type, but other structures resulting from ester-amine polycondensation reactions have been included. Carrier anchoring of the ferrocenylation agent of choice, 4-ferrocenylbutanoic acid, is brought about both by acylation of carrier-attached amino groups, leading to amide links in the spacer, and by acylation of polymer-bound hydroxy groups, resulting in ester linking of the ferrocene unit. Selected conjugates are being screened in cell culture tests for antiproliferative activity against the HeLa and LNCaP human cancer lines, and preliminary results are highly promising, with IC_{50} values in the representative range of 2-20 µg Fe/mℓ. In view of the relatively low level of toxic side effects expected for these organoiron compounds, the findings here presented, however limited in scope, offer challenging opportunities for the development of iron-containing, polymer-anchored drug systems as chemotherapeutic agents in cancer research.

1 Ferrocene in the biological environment

The organoiron compound ferrocene, di(η^5-cyclopentadienyl)iron(II), has, since its discovery in 1951, provided a powerful impetus to basic and applied research activities in the broad field of organometallic chemistry, with numerous ramifications into the chemistry, physics, and technology of metal-containing systems. The behavior of the ferrocene complex in the biological environment has in more recent years been identified as an eminently challenging,

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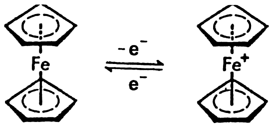
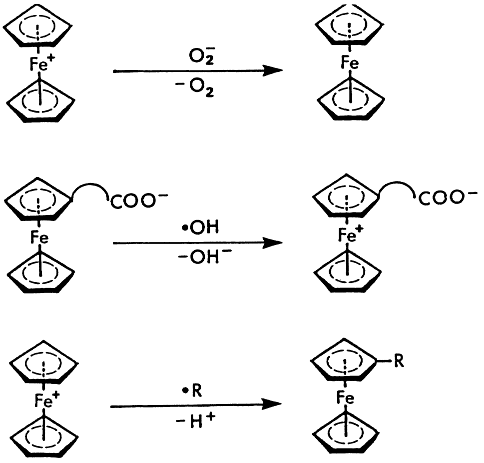
proving ground for fundamental investigations and, in particular, for research focusing on the chemotherapy of cancer.

The neutral ferrocene complex readily undergoes one-electron oxidation, transforming to the ferricenium cation (Scheme 1):



Scheme 1

The step involves removal of an electron from one of the highest-lying, essentially non-bonding a_1 and e_2 orbitals, leaving an unpaired electron on the metal and so rendering the oxidation product a free-radical species of appreciable stability. Many biological interactions involve free-radical chemistry, frequently with participation of reactive oxygen species, and the ferrocene/ferricenium couple, when placed into such biological environment, can be expected to play an active part in it. Thus, ferricenium interaction with the superoxide radical anion, an important biological role-player, leads to reduction of the cation with concomitant superoxide conversion to dioxygen[1]. Further, certain ferrocenylcarboxylates may react with the deleterious hydroxyl radical, which is transformed in the process to the hydroxide anion[2]. The ferricenium cation may also undergo simple recombination with other potentially destructive free radical species, followed by proton elimination, the net result being the generation of a neutral ferrocene derivative[3]. These reactions, depicted in Scheme 2 (top to bottom), evidently represent potential detoxification paths as deleterious oxygen radical species are removed from the biological system.



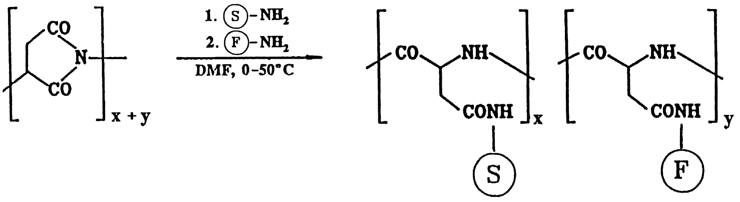
Scheme 2

Free radicals play key roles in many stages of carcinogenesis and in the progression and containment of neoplasias. Grounded on our hypothesis, however vague at that time, that the ferrocene/ferricenium couple might lend itself as an active interceptor in such processes, a program was initiated in the author's laboratory some two decades ago, with the objective of exploring the potential of ferrocene action in the transformed cell.

A large number of ferrocene and ferricenium compounds was prepared, compositionally characterized[4], and, in selected cases, tested for antiproliferative and antineoplastic activity in collaboration with overseas institutions[5-8]. A key finding in that initial work was the requirement of water solubility. Ferrocene itself, but sparingly soluble in water, was inactive. In contrast, water-soluble derivatives, notably the ionic ferricenium salts with reasonably non-hydrophobic counter-ions, such as tetrachloroferrate, μ-oxo-bis(trichloroferrate), picrate, and trichloroacetate, displayed good to excellent performance both *in vitro* and, where selected for that purpose, also *in vivo*. We have argued[9] that for a systemically administered ferrocene drug model the *in vivo* equilibrium concentrations of ferrocene and ferricenium species depend solely on the instantaneous biological environment, such as pH and enzyme activities, and not on the initial oxidation state of the administered dosis form, provided only that the latter is a water-soluble system for efficacious distribution in central circulation.

2 Polymer-ferrocene conjugation : earlier studies

Prompted by these observations in the realm of nonpolymeric ferrocenes, a follow-on program aiming at the development of carrier-bound ferrocene compounds was set up. The reversible binding (anchoring) of a medicinal agent to a water-soluble carrier polymer, meeting certain biomedical specifications, such as biocompatibility and biodegradability, can lead to considerably enhanced therapeutic effectiveness, as crucially important factors combine to increase bioavailability at the target site while reducing toxicity and risk of resistance build-up. Following Ringsdorf's pioneering paper[10], the carrier-anchoring concept has been utilized in numerous laboratories, and the topic has been treated in exemplifying reviews[11-13]. Anchoring the ferrocene unit to such water-soluble carriers should provide the very conditions that would enable the metallocene to expand its potential as a drug model and chemotherapeutic agent. Exploratory work, screening a variety of ferrocene compounds as candidate models for carrier anchoring, indeed established the feasibility of polymer anchoring, yielding water-soluble polymer-ferrocene conjugates in which synthetic polyamides served as the carrier components[9,14]. Specifically, the carriers were of the α,β-poly(DL-aspartamide) type, synthesized from polysuccinimide by an aminolytic ring-opening process pioneered by Neri *et al.*[15] and further developed by Drobnik *et al.*[16]. In our laboratory a two- or three-step procedure was elaborated allowing for two or more different amines to be introduced in given molar ratios (Scheme 3; only the α-peptidic form is shown here and in subsequent representations).



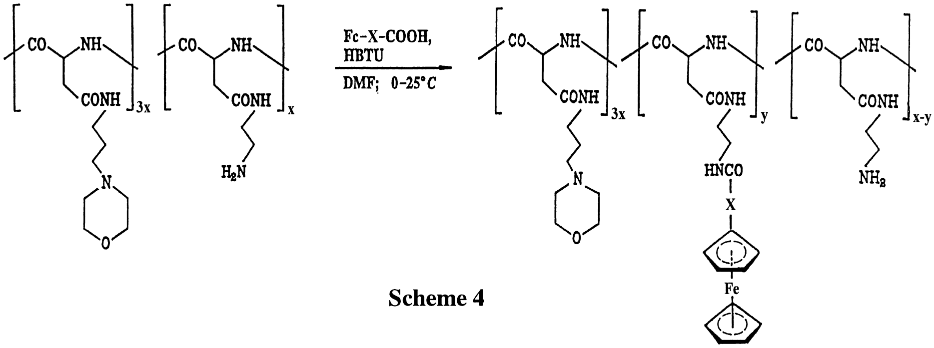
Scheme 3

The procedure permits the formation of carriers comprising a major proportion of solubilizing units and a minor fraction of units functionalized for drug binding. The latter may typically contain a primary amino group terminal in **F**, whereas the former may feature a hydroxy or tertiary amine terminal in **S**. The random α,β-DL-configuration in these polyamides provides an ideal backbone structure. While prone to slow *in vivo* fragmentation as required for ultimate excretion, it is unable to undergo α-peptidase-mediated, unduly rapid "unzipping" of the chain, which would result in premature termination of the carrier function. Furthermore, the weight-average molecular weight, controlled by dialysis, generally ranges from about 20 000 to 30 000. This range is too low to cause polymer-associated toxic effects, yet high enough to retard renal clearance. Lastly, the versatility inherent in the multistep synthesis method permits the design and construction of chains in a predetermined variety of subunit composition and frequency.

Among the various compounds employed as ferrocenylation agents, ferrocenylcarboxylic acids proved to be the most useful ones, largely on electrochemical grounds[17,18]. 4-Ferrocenylbutanoic acid, having a favorable formal reduction potential less positive than that of ferrocene proper, was selected as the agent of choice.

The anchoring reactions, involving amino side group acylation, were performed by treating the amine-functionalized poly(aspartamide) carriers in dimethylformamide solution with the ferrocenylation agent in the presence of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylisouronium hexafluorophosphate (HBTU) coupling agent. Alternatively, the ferrocenylbutanoic acid was first converted to its active succinimidyl ester, which was then allowed to react with the carrier, again in dimethylformamide solution. The ferrocene/NH_2 molar feed ratio was 1.5 in both methods. The water-soluble reaction products were purified by aqueous dialysis and collected upon freeze-drying as solid polymers with iron contents typically ranging from 2 to 5 %. Degrees of N-acylation remained slightly short of the 100%

mark; in the exemplifying conjugate structure **2** (Scheme 4), y/x ratios of 0.93 – 0.98 may be considered representative.



Scheme 4

3 Polymer-ferrocene conjugates : recent developments

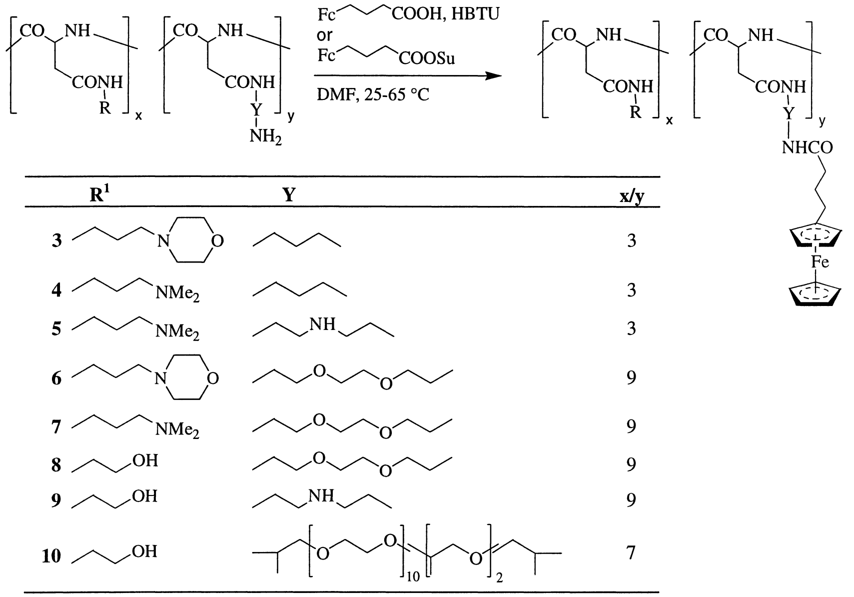
With the ground work laid in the earlier projects, the scope of the ferrocene anchoring program has more recently been widened to include a greater number of carrier types. Furthermore, in addition to the ferrocene anchoring mechanism involving carboxamide linking, carrier binding *via* ester links has been investigated. The two different anchoring modes will be discussed separately in the following two sections.

3.1 Carboxamide-linked conjugates

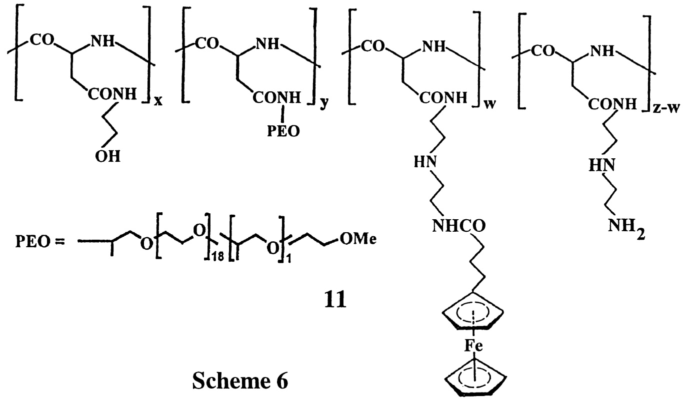
This drug-linking mode, utilized in previous work and commented upon in the foregoing section, provides a link that is biocleavable predominantly by proteolytic enzyme action in the lysosomal compartment, while reasonably resistant to hydrolysis at physiological pH. The anchoring methodology used for the N-acylation reactions reported in this section has been substantially the same as in the earlier work. Where needed, however, slightly more forcing conditions, such as a ferrocene/NH_2 molar feed ratio increased to 1.8 and the occasional inclusion of a re-acylation treatment, have been introduced in order to achieve complete substitution of available amino groups. As in the earlier work, suitably amine-functionalized poly(aspartamide)s have served, and continue to serve, as the “work horse” carrier type. Scheme 5 depicts the formation of a representative collection of conjugates from their respective carriers[19,20]. Polymers **3** – **10** are soluble in water and possess iron contents of about 2-10 %.

Whereas compound **10** comprises a poly(ethylene oxide) chain in the spacer segment, a conjugate featuring a poly(ethylene oxide) graft in the overall recurring unit is exemplified by

11, with a ratio of x/y/w/z-w = 6.42 : 0.67 : 1 : 0.25 (Scheme 6). The conjugate is prepared by ferrocenylation of a terpolymer carrier containing the anchoring amine function in the z-subunit.



Scheme 5



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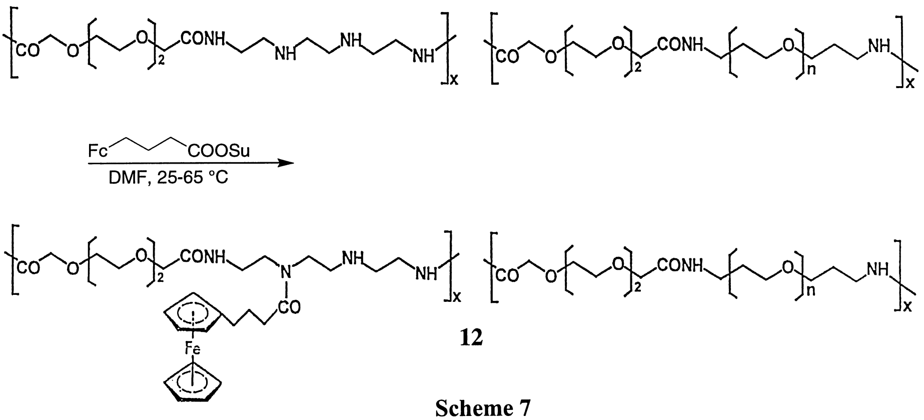
Scheme 6

The conjugates presented in the foregoing feature more than 10 atomic constituents in the spacer connecting the metallocene with the main chain of the carrier, thus allowing for a certain measure of steric access to approaching enzymes. It should be of interest, for comparison of potentially different pharmacokinetics, to prepare conjugates possessing distinctly shorter spacer segments such as would result from anchoring of the ferrocenylation agent directly to the main chain. This concept has been realized through ferrocenylation, by the active ester method, of linear polyamides that contain secondary amino groups as backbone constituents, thus permitting direct main-chain acylation[21]. The approach is exemplified in Scheme 7 for the ferrocenylation of a carrier type prepared by an ester-amine polycondensation reaction explored previously in this laboratory. The molar feed ratios of metallocene per carrier repeat unit, ranging from 1.4 to 1.6, have been selected here so as to allow just one ferrocene group to be anchored in the recurring unit, giving the water-soluble conjugates **12**, where n = 3 or 21. A special feature of these polymers, and numerous related conjugate structures not shown, is the inclusion of oligo(ethylene oxide) segments. Ethylene oxide polymers are known for their contribution to solubility, reduction of protein binding, and other biomedically desirable properties.



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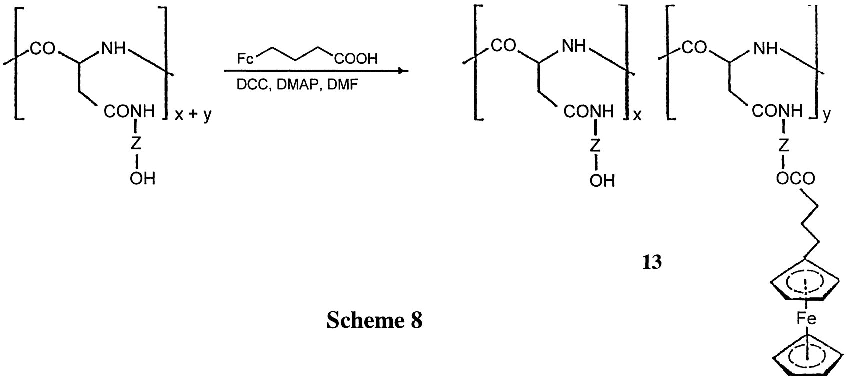
Scheme 7

3.2 Ester-linked conjugates

Drug conjugation *via* ester linking can conveniently be achieved by esterification of a hydroxy-functionalized carrier with a carboxylic acid form of the drug[22]. Using 4-ferrocenylbutanoic acid as the ferrocenylation agent and poly(aspartamide)s containing side hydroxy groups, the conjugation step is brought about, again in dimethylformamide solution, through mediation of dicyclohexylcarbodiimide coupling agent in the presence of 4-(dimethylamino)pyridine as a catalyst. The exemplifying conjugates **13** shown in Scheme 8 (R = $(CH_2)_2$ or $(CH_2)_2O(CH_2)_2$), purified as before by dialysis, are isolated upon freeze-drying as water-soluble polymers. With molar feed ratios of ferrocenylation agent per carrier repeat unit in the range of 0.25 – 0.5, ^{1}H NMR spectroscopic data show the x/y ratios in the resulting structures **13** to range conveniently from approximately 3 to 10, corresponding to iron contents of about 2.5 – 5.5 %.

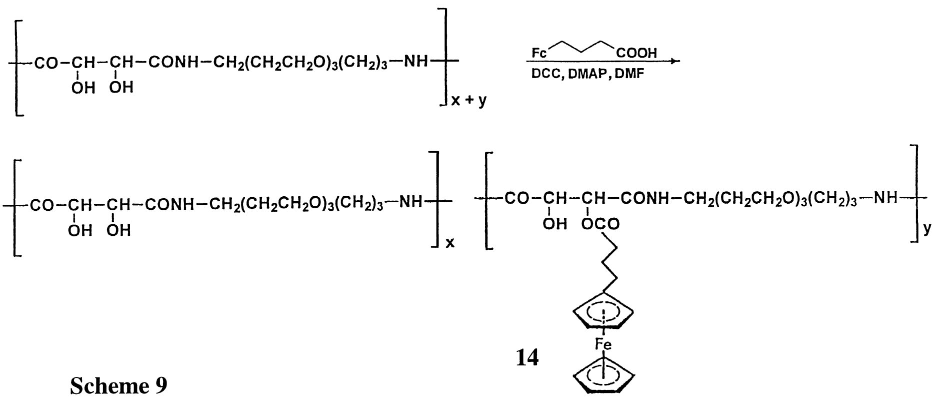
A conjugate type containing the drug model in a location of shorter distance from the main chain results from esterification of carriers containing hydroxy groups directly bonded to the backbone as exemplified by the structure shown in Scheme 9.



13

Scheme 8

Polyamides of this type are accessible by ester-amine polycondensation of diethyl tartrate with amine-terminated ethylene oxide oligomers. With 4-ferrocenylbutanoic acid as the ferrocenylation agent, esterification under conditions similar to those outlined in the foregoing provides water-soluble conjugates **14** and related structures with compositions typically corresponding to x/y = 4 and iron contents of 3 %.



14

Scheme 9

4 Cell Culture Testing

In order to arrive at a tentative assessment of cancerostatic properties of the various categories of ferrocene-containing polymers developed in this laboratory, a first-round *in vitro* screening program was recently executed (since published[20]), in which several representative polyaspartamide-ferrocene conjugates were tested against the HeLa human cervix epitheloid carcinoma cell line. The project included the compounds **3**, **4**, and **7** containing comparatively short spacer segments. Further included were the poly(ethylene oxide)-modified conjugates **10** and **11**. From plots of cell growth (relative to untreated control) *versus* conjugate concentration, reproduced in Fig. 1, IC_{50} values were determined, expressed here in terms of iron concentrations required for 50% cell growth inhibition.

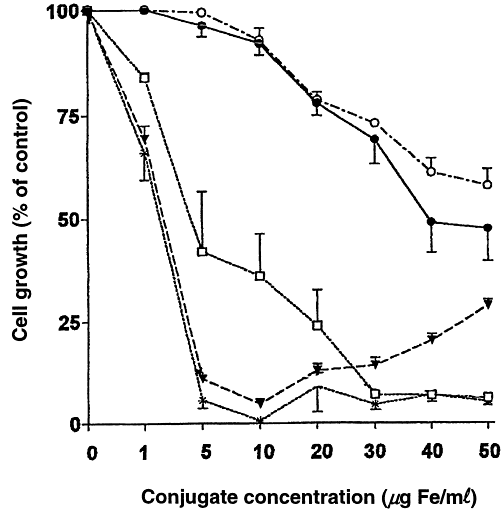


Figure 1 Cell growth in % of control *versus* conjugate concentration in terms of μg Fe/ ml. (* **4**, ▼ **7**, □ **3**, ● **10**, ○ **11**)

As can be seen from the graphs, the first three conjugates enumerated show remarkably high antiproliferative activity, best performance being displayed by **4** and **7**, with $IC_{50} \approx 2$ μg Fe/ml. Distinctly higher values, in the proximity of 50-60 μg Fe/ml, were determined for the remaining two polymers **10** and **11**. Their somewhat inferior performance may possibly be associated with the presence of poly(ethylene oxide) segments causing diminished steric accessibility to the lysosomal enzymes active in the spacer-cleaving step. It might also be argued that **3**, **4**, and **7**, contrasting with **10** and **11**, are characterized by the incorporation of tertiary amine functions which, being powerful proton acceptors, may impart cationic behavior at physiological pH and below. This would facilitate adsorptive pinocytotic cell entry of the conjugates as observed, for example, with polylysine. Other evidence to be discussed further below, however, renders this argument somewhat less convincing.

In a follow-on screening project, still in progress[23], the same conjugates are being tested for antiproliferative activity against the LNCaP human metastatic prostate adenocarcinoma cell line. The list of candidate conjugates for screening additionally includes the related structures **5** and **6**, as well as the two conjugates **8** and **9** containing hydroxy group in place of tertiary amine side group terminals. Provisional IC_{50} data, summarized in Table 1, indicate a comparable performance trend against both cell lines for the retested **3**, **4**, **7**, **10**, and **11**, with IC_{50} in the range of 2-5 μg Fe/ml for **3**, **4**, and **7**, and tentatively at 35 μg Fe/ml and higher for **10** and **11**. Very good, though equally tentative, IC_{50} values are also apparent for **5** and **6**. Significantly, the two hydroxy-modified structures **8** and **9** give IC_{50} values (10 and 3 μg Fe/ml) substantially at the same general level of the tertiary amine-modified polymers. Both **8** and **9** are unable to convert to cationic species under *in vivo* conditions; the found values, hence, suggest that, at least on the basis of the limited number of compounds so far tested, the lower activity previously observed for **10** and **11** is most likely due to different factors, such as the presence of poly(ethylene oxide) components, as was pointed out before.

Table 1 Antiproliferative activity of selected poly(aspartamide) - ferrocene conjugates

Conjugate	% Fe, nominal	Base molecular weight[a]	IC_{50}[b]	
			HeLa[c]	LNCaP[d]
3	4.9	1149	7.2	5
4	5.5	1023	2.1	3
5	5.3	1052	-	2
6	2.1	2671	-	6
7	2.4	2293	2.3	2
8	2.9	1923	-	10
9	3.0	1878	-	3
10	2.7	2099	45	35
11	2.5	2240	60[e]	> 65

[a] Molecular weight of simplest recurring unit. [b] Conjugate concentration, expressed as μg Fe/ml, required to achieve 50% cell growth inhibition (relative to untreated control). [c] HeLa human cervix epitheloid carcinoma[20]. [d] LNCaP human metastatic prostate ardenocarcinoma[23]. [e] Estimated by extrapolation beyond 50 μg Fe/ml.

Considering now the IC_{50} data (not tabulated) provided for a limited number of polymers of the type **13** with ester-bound ferrocene, we find these to fall within the 5-35 μg Fe/ml range, roughly equivalent to, or just insignificantly higher than, those of the amide-bound polymers. Whether the absence of potentially cationic sites in this class of conjugates could have affected the observed activity remains an open question at this time, and a forthcoming, considerably broadened testing program is expected to shed further light on that issue. What does emerge from these findings at the present state of *in vitro* screening is the apparent lack of significant acceleration of drug release in the ester bond, relative to the amide link; *in vivo* tests, however, may well lead to a different conclusion.

Somewhat anomalous is the observation that for several polymers the curves, allowing for promising IC_{50} values in the initial stage of low drug concentrations, tend to approach a plateau well above the zero cell growth level at further increased concentrations. In the case of **7**, the curve even re-ascends at higher conjugate concentrations, indicating an increase in cell survival as drug concentrations are raised further, and this trend holds for the screens against both cell lines. This phenomenon needs further scrutiny.

5 Future Work

Although the *in vitro* test results so far obtained are provisional and require corroboration on a significantly broadened scale in forthcoming screening work, they clearly confirm the early expectations of carcinostatic properties of suitably designed polymer-anchored ferrocene compounds possessing water solubility. Future investigations must address a number of questions. Thus, it must be determined whether conjugates incorporating the ferrocene system in the oxidized (ferricenium) state will, indeed, show a performance spectrum similar (and not superior) to that of the neutral ferrocene analogs. This should serve to confirm our view that, especially under *in vivo* conditions, the ferrocene/ferricenium equilibrium concentrations will be controlled entirely by the local biological environment and not by the oxidation state in which the conjugates are administered. Another problem concerns the "aging" effect frequently observed in this laboratory with polymer-anchored ferrocene compounds, resulting in slow and gradual loss of water solubility as a consequence of intermolecular solid-state interaction at room temperature. Although storage in the frozen state alleviates this problem, further action, such as embedding in water-soluble matrices, is needed to increase intermolecular distances in the solid state. Future work will also assess the influence of molecular weight on biological performance and the effect of such structural factors as the nature of the **S** and **F** groups (Scheme 2) and of the spacer segments, the latter to include oligopeptide components[12]. In addition, carrier structures other than those discussed in the foregoing will be included. The kinetics of drug release and carrier degradation require attention, and so do the topics of biodistribution and bioavailability in the neoplastic tissue. Lastly, and most importantly, relative conjugate activities against drug-resistant cancer lines must be determined in comparative studies in an effort to identify structural factors that might be exploited for the eventual circumvention of multidrug resistance.

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