Abstract. Background: Apoptotic propensity is currently viewed as an important parameter in drug-induced toxicity. But other cell death pathways exist e.g. micronucleation, intermitotic cell death, abnormal nuclear morphology and necrosis. This investigation explores the onset of apoptosis and abnormal morphology in response to 3 drugs i.e. Cisplatin, a novel Ferrocene (fctfa) and a novel Rhodium-Ferrocene [Rh(fctfa)(cod)] complex. Materials and Methods: A pair of prostate cell lines from normal human prostate epithelium (1542N) and malignant human prostate epithelium (1542T) were exposed to increasing concentrations of the drugs for 24 hours, double-stained with FITC-AnnexinV and with Propidium Iodide and analysed by dual parameter flow cytometry to quantitate viable cells in quadrant I, early apoptotic cells in quadrant IV and late apoptotic/necrotic cells in quadrant III. Apoptosis was also scored by microscopy after Acridine Orange staining, by Western blots for caspase 3 induction and for caspase 8 induction using a colorimetric assay. Results: The toxicity of Cisplatin and the novel Ferrocene and the novel Rhodium-Ferrocene complexes was found to be 0.9-1.3 µM; 4.1-4.5 µM and 10.1-13.2 µM, respectively. Apoptotic propensity scored after 24 hours was found to be dose-dependent and in the range of 7-19 % for Cisplatin and 1-4.1 % for the Ferrocene and Rhodium-Ferrocene complexes. Cisplatin produces a distinct apoptotic response followed by a necrotic response, whereas the Ferrocene and the Rhodium-Ferrocene complexes produce a massive necrotic reaction in the region of 3-19 % and very little if any apoptosis. Absence of apoptosis was corroborated by lack of caspase 3 activation, absence of typical apoptotic morphology and by lack of caspase 8 activation. Conclusion: The 3 drugs Cisplatin, the novel Ferrocene and the novel Rhodium-Ferrocene complexes show similar toxicities in the 1-10 micro-molar range in prostate cell lines. However the drugs differ significantly in the activation of death pathways. While Cisplatin predominantly induces apoptosis documented by morphology, Annexin V staining and caspase 8 activation, the Ferrocene and Rhodium-Ferrocene complexes induce late necrosis and abnormal nuclear morphology. Unlike Cisplatin-treated cells which enter apoptosis and necrosis sequentially, the 2 Ferrocene drugs invoke direct entry of cells into late necrosis without first entering the early apoptotic compartment.

In the search for new antitumour drugs we have evaluated ferrocenyltrifluoroacetone here called Ferrocene and (n-4 1,5 cyclooctadiene) (1-ferrocenyl-4,4,4-trifluoro-1,3-butanedionato-k2 O,O') rhodium (I) here called Rhodium-Ferrocene against a range of prostate cell lines for which the sensitivity against established antitumour drugs was known (1,2). The chemical synthesis and solubility properties of these drugs have been described elsewhere (3-6). In the current in vitro study clarification was sought about the effectiveness of IC50 concentrations of Ferrocene and Rhodium-Ferrocene against a range of prostate cell lines for which the sensitivity against established antitumour drugs was known (1,2). The chemical synthesis and solubility properties of these drugs have been described elsewhere (3-6). In the current in vitro study clarification was sought about the effectiveness of IC50 concentrations of Ferrocene and Rhodium-Ferrocene against normal and tumour cells of prostatic origin in relation to Cisplatin as the reference drug. Mechanistic aspects of drug toxicity were evaluated by measuring apoptotic and necrotic propensity to trace the induced cell death pathways and drug specific response patterns.

Materials and Methods

Cell culture maintenance. Cells were maintained at 37°C in 95% air and 5% CO2 in a humidified atmosphere. Seeded cells were left to attach to the surface of the cell culture flasks for at least 6 hours before the onset of experiments.
Drugs. Drug dilutions were generated as follows: 20 mg Rh-Ferrocene (MW 534.1) was dissolved in 10 ml of dimethyl sulfoxide (DMSO) and filtered through a 0.22 mm filter into a sterile culture tube using a syringe (stock I). 1 ml of stock I solution was added to 9 ml of growth medium to generate a 1 mM drug solution (stock II). Stock II was used to generate the required drug dose points in the mM range.

Drug toxicity. Exponentially growing cells were trypsinised, counted and 10,000 cells/ml seeded into 24-well multiwell plates. After 6 hours freshly prepared drug dilutions were added and cells were incubated for 24 hours when the medium was changed and the multiwell plates re-incubated for a further 4 days. The cells were then fixed in buffered formalin (pH 7.2) and stained with 0.1% crystal violet. The crystal violet stain was then extracted with 10% sodium dodecyl sulphate (SDS) overnight and read at 590 nm.

Apoptosis measurements. Apoptosis measurements were performed by double staining with the Annexin V-Fluos reagent and Propidium Iodide (PI) in binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl and 5 mM CaCl2). Analysis was performed on a FACScan flow cytometer at 488 nm using a 515 nm bandpass filter for fluorescein detection, and a bandpass filter >560 nm for PI detection. Dot plots in log scale of FL-1 vs. FL-3 were used to identify the live (Annexin V- and PI-negative) population, as well as early apoptotic (Annexin V-positive, PI-negative), late apoptotic (Annexin V- and PI-positive), and necrotic (Annexin V-negative, PI-positive) populations delineating the respective populations in 4 quadrants as shown in Figure 2.

Gel electrophoresis. Electrophoretic separation of cell lysate proteins was on SDS gels using a 12% separating gel and a 4.5% stacking gel and 10-250 kD molecular weight markers (Biorad, USA), essentially as described by Laemmli (10). Gels were run for approximately 50 minutes at 200 V until the dye-front reached the end of the gel.

Caspase-3 assays / Western blots. Proteins were transferred by electroblotting in: glycine (192 mM), Tris-HCl (10 mM) and methanol (20%), at 200 mA for 1 hour at room temperature. Even loading of samples was confirmed by Ponceau Red (Sigma, Germany) staining. Membranes were pre-soaked in methanol for 30 seconds and then rinsed in transfer buffer. The membrane was then blocked in Tris-buffered saline-Tween 20 (TBST) containing 10% blocking agent.
Figure 2. Progression of cell death in 1542 tumor cells after exposure to cisplatin for 24 hours. A: controls, B: 3μM, C: 30μM, D: 180μM
Rapid increase of Annexin-positive cells in quadrant IV from 3 to 19% indicates program of early apoptosis. Increase of Annexin- and PI-positive cells in quadrant III from 6 to 64% indicates induced levels of late apoptosis/necrosis. Possibility exists that early apoptotic cells proceed to late apoptotic/necrotic stage. The possibility of viable cells entering necrosis directly, without going through apoptosis is very real. See control panel A.
non-fat milk powder, for 2 hours at room temperature, washed 5x in Tris buffered saline (TBS)/Tween 20 solution followed by rotating overnight at 4°C. Incubation with anti-caspase-3 antibody (PharMingen, USA) was followed by several washes in TBS/Tween 20 solution before addition of the anti-mouse IgG horse radish peroxidase antibody (Amersham, England) and incubation for 1 hour at room temperature. The membrane was then washed several times in TBS/Tween 20 solution. After incubation for 60 seconds with the ECL (Amersham) reagents (luminol and horse radish peroxidase) the membrane was cling wrapped and exposed to Hyperfilm ECL (Amersham) for 1-30 minutes.

Data evaluation. All experiments were repeated at least three times to calculate the mean and standard deviation. Statistical analysis and date fitting was by GraphPad Prism (GraphPad software, San Diego, USA). A two-sided t-test was used to compare means between sample groups.

Results

The molecular structures of Ferrocene and Rhodium-Ferrocene (Figure 1) illustrate novel substitutions on the Ferrocene skeleton to generate new antitumour drugs. The toxicity of the 2 drugs for prostate tumour cells are in the micromolar range and are similar to Cisplatin (Table I). In 1542 tumour cells the 2 drugs are 4x less effective than Cisplatin, while in 1542 normal prostate cells the toxicity is approximately 10x lower. This suggests that normal non-malignant prostate cells undergo a more selective protection when treated with either of the 2 Ferrocene drugs than with Cisplatin (Table I).

Analysis of the progression of cell inactivation shows that prostate tumour cells rapidly enter apoptosis and late apoptosis/necrosis at very low Cisplatin concentrations (Figures 2, 3). Increase of the Cisplatin concentration is shown to give rise to a rapid increase of Annexin-positive cells in quadrant IV (0.4-3.1%) indicating low apoptotic propensity. High abundance quadrant III even at 6 hours suggests rapid entry of cells into the late apoptosis/necrotic pathway without first entering early apoptosis.

Table I. IC_{50} (µM) for Cisplatin, Ferrocene and Rhodium-Ferrocene, determined by MTT assay.

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<th>Cell lines</th>
<th>Ferrocene</th>
<th>Rh-Ferrocene</th>
<th>Cisplatin</th>
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<td>1542T</td>
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<td>0.88</td>
</tr>
<tr>
<td>1542N</td>
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<tr>
<td>Caco-2</td>
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Figure 3. Progression of cell death in 1542T cells exposed to 25µM FeTA and 10µM RhFe for 6 and 12 hours. Very low abundance of Annexin V-positive cells in quadrant IV (0.4-3.1%) indicates low apoptotic propensity. High abundance quadrant III even at 6 hours suggests rapid entry of cells into the late apoptosis/necrotic pathway without first entering early apoptosis.
Figure 4. Response of prostate cell lines after 24-hour exposure to cisplatin.
A: 1542N cells, B: 1542T cells, C: 1542T cells and Ferrocene, D: 1542T cells and Rh-Ferrocene determined by FITC Annexin V and PI double-staining and flow cytometry.

- O--O viable cells from quadrant I
- Δ--Δ early apoptotic cells from quadrant IV
- □--□ late apoptotic/necrotic cells from quadrant III
Similar experiments with Ferrocene and Rhodium-Ferrocene show that these 2 drugs generate very little, if any, apoptotic cohorts in quadrant IV but substantial populations of late necrotic cells in quadrant III (Figure 3). A time study at comparatively low drug concentrations shows that the 6- and 12-hour exposure to 25 μM Ferrocene and 10 μM Rhodium-Ferrocene indeed only generate 0.4-2.4% of Annexin V-positive cells in quadrant IV, indicating a very low apoptotic response to the 2 drugs. Interestingly, the late apoptotic population in quadrant III increased rapidly with time to the level of 5% suggesting direct entry of cells into necrosis, without moving first into apoptosis (Figure 3).

The decline of viable cells in quadrant I and the appearance of apoptotic and necrotic cells in quadrants IV and III shows that prostate cells indeed react differently to the 3 drugs. Whereas Cisplatin produces a distinct apoptotic (quadrant IV) response followed by a necrotic (quadrant III) response (Figures 4A, B), Ferrocene and Rhodium-Ferrocene given to the same 1542 tumour cell line produce a massive necrotic reaction and a very low apoptotic reaction (Figure 4C, D) which remains in the region of 2% (Table II) in the 2 prostatic cell lines.

A low apoptotic response to the 2 new anticancer drugs in the region of 2% was also indicated by microscopic analysis after Acridine Orange staining (Table II) and is consistent with caspase 3 induction (Figure 5). Western blots and densitometric ratios relative to untreated controls show that caspase 3 expression at a drug concentration of 10 x IC50 remain in the region of 1-1.5 for both drugs (Figure 5A, B). The caspase-8 data assayed 48 hours and 72 hours after...
treatment with Ferrocene and Rhodium-Ferrocene show only a 0.4-1.6-fold and 1.0-1.28-fold increase, respectively over untreated controls, whereas Cisplatin brings the caspase 8 expression to a 2-3.3-fold increase in both time frames (Table III). The much lower caspase 8 expression for both Ferrocene and Rhodium-Ferrocene as compared to Cisplatin is consistent with the AnnexinV data and morphological evidence (Table II), which also indicated a significantly more muted apoptotic response for the new drugs. A semi-quantitative morphological analysis of aberrant cell types produced by the 3 drugs indicated that Ferrocene and Rhodium-Ferrocene produced large cell populations with abnormal morphology, such as large nuclei, micronuclei, aneuploidy and tetraploidy (Figure 5). In the Cisplatin-treated cells apoptotic cells were much more numerous (not shown). These observations would support the conclusion that the death pathways mobilised by Ferrocene and Rhodium-Ferrocene differ from the death pathways induced by Cisplatin.

**Discussion**

The toxicity measurements using vital dye staining assays (11,12) indicate IC50 values in the range of 1.0 for Cisplatin and 4-13 for FeTA and RhFe, depending on the cell line (Table I). While the lower toxicity of Ferrocene and Rhodium-Ferrocene may seem disadvantageous from the clinical point of view, the 2 drugs clearly show new and interesting properties in the damage and death pathways. Cisplatin induces substantial levels of apoptosis, as indicated by Annexin V binding, microscopic scoring after Acridine Orange staining and induction of caspases 3 and 8. The 2 Rhodium drugs produce essentially very little, if any, apoptosis but large necrotic populations. The necrotic cells are identified on the basis of positive PI staining in aqueous milieu, hence indicating deteriorated nuclear membranes. The abnormal morphology of cells is characterized by nuclear deterioration mainly consisting of aneuploidy and nuclear aggregates and hence abnormal karyokinesis. It therefore seems that the late apoptotic/necrotic cells and the cells with abnormal morphology may be closely related, if not the same. The accumulation of large proportions of necrotic cells detected by leaky nuclear membranes and positive PI staining, and the concurrent increase of cells with abnormal morphology strongly suggests this. That abnormal morphology is a feature of cellular damage and expression of a separate cell death pathway has previously been suggested from the irradiation response of L929 mouse fibrosarcoma, HaCat human keratinocytes, colo-320 human liver carcinoma and K562 human myeloid leukemia cells (13), where the percentage of abnormal cells accumulating after irradiation damage was found to be dose- and cell line-dependent, varying from 5% to nearly 60%, often far exceeding the proportions of apoptotic

<table>
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<th>Rhodium-Ferrocene</th>
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<tr>
<td>1542T</td>
<td>2.13</td>
<td>1.60</td>
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<tr>
<td>1542N</td>
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*) The fold increase after 72 hours of drug exposure was 1.33-3.40 for Cisplatin and 0.96-1.28 for Ferrocene and 0.41-0.81 for Rhodium-Ferrocene.
cells and the micronucleated cells. The existence of death responses other than micronuclei and apoptosis was also postulated from the failure of reconstructing radiosensitivity (cell survival) from apoptosis and micronucleus frequencies alone. This missing parameter, termed probability of unknown events (Poe), was found to be cell line- and dose-dependent (14,15). The evidence for the existence of alternative death pathways thus is very strong. The abundance of a substantial population of necrotic cells in the controls which increases upon infliction of damage, without giving rise to increase of apoptotic cells (Figure 3), is a strong indication of the existence of a direct pathway into necrosis and abnormal morphology. Other observations on the 1542 prostate cell line also suggest the possibility of an independent pathway to necrosis, not including apoptosis (7). That apoptosis could be overrated in importance as a major death pathway is now being seriously debated (9,16-19), strong arguments against apoptosis coming from experiments on gastric tumour cells showing that irradiation doses which remove 96 % of mitotically-competent cells only generate 10 % apoptotic cells (17). Processes which profoundly modify apoptotic propensity have been found to have no effect on cell survival (18). In a major review of the apoptotic route in the mechanism of damage-induced cell death, Steel comes to the conclusion that apoptosis is not the only pathway of cell death and may operate downstream of critical events which precipitate mitotic failure and loss of reproductive integrity (16). Our demonstration that the 2 Ferrocene drugs and Cisplatin invoke different death responses in the same cell line indicates that such responses can be drug specific. While the cytotoxicities of Cisplatin, Ferrocene and Rhodium-ferrocen in prostate cells are similar we find that the death pathways are not. Whether this differential mechanistic behavior of the 2 Ferrocene drugs is of any clinical benefit remains to be seen. The possibility clearly exists that apoptosis resistant cells can be better killed with drugs inducing the necrotic pathway. The overriding outcome of our results is that apoptosis is not the only natural process of cell elimination. Further experimentation would be required to test the validity of the specific action of Ferrocene and Rhodium-Ferrocene in other cell lines and especially in cells lines of high apoptotic propensity.

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References


