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Short communication

Influence of methoxy groups on the antiproliferative effects of [Fe^{III}(salophene-OMe)Cl] complexes

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ABSTRACT

We synthesized methoxy-substituted iron(III)-salophene complexes ([Fe^{III}(OMe-salophene)Cl] with salophene = *N*,*N*′-bis(salicylidene)-1,2-phenylenediamine) and analyzed their biological activity in MCF-7 and MDA-MB-231 breast cancer as well as in HT-29 colon carcinoma cells. The results obtained in a time-dependent chemosensitivity test clearly demonstrated the correlation between the cytotoxicity of the complexes and the position of methoxy substituents in the salicylidene moieties: 3-OCH_3 (**4**) < 5-OCH_3 (**8**) < H (**2**) < 4-OCH_3 (**6**) = 6-OCH_3 (**10**). Compounds **6** and **10** caused cytocidal effects already at a concentration of 0.5 μM. Both lead compound **2** and complex **8** showed similar time response curves, however, with a 5-fold lower activity compared to **6** and **10**, respectively. Referring to [Fe^{III}(salophene)Cl] (**2**), methoxy substitution was accompanied with the loss of tumor cell selectivity. Moreover, the free ligands (**1**, **3**, **5**, **7**, and **9**) were inactive.

1. Introduction

The discovery of the inorganic complex cisplatin revolutionized the treatment of testicular cancer and led to increased research of organometallic complexes as antitumor agents [1]. Among them, a variety of iron containing compounds has emerged due to their outstanding biological properties. The group of Neuse reported about antitumor activity of a series of ferrocene complexes against Ehrlich ascites tumor in CF1 mice [2,3]. Moreover, according to the findings of James et al. enantioselective ferrocenyl nucleoside analogues turned out to be apoptosis-inducing compounds in Burkitt-like lymphoma (BJAB) tumor cells [4]. Hillard et al. developed ferrocenyl complexes combining antiestrogenicity and estrogen-independent cytotoxicity in the same molecule in order to address both estrogen receptor positive and negative breast cancer cells [5]. Furthermore, it was demonstrated that the DNA cleavage ability of hydroxyl-substituted iron-salen complexes (salen = *N*,*N*′-bis(salicylidene)ethylenediamine) strongly depended on the position of the hydroxyl groups in the salicylidene moiety [6]. The group of Tumminello postulated a non-intercalative mode of action with DNA of iron-salen complexes, although the flat molecules can be stacked between two base pairs. They confirmed an external electrostatic interaction between the negatively charged DNA double helix analogously to porphyrazines and metal–porphyrazine complexes [7]. This might induce apoptosis via mitochondrial pathway as demonstrated for human HEK-293 cells [8].

In a previously published structure–activity relationship study we described the development of [*N*,*N*′-bis(salicylidene)-1,2-phenylenediamine]iron(III) ([Fe^{III}(salophene)Cl]) as antitumor agent and the evaluation of the mode of action [9]. The same lead structure was used by Lange et al. [10] and they demonstrated a selective action against platinum-resistant ovarian cancer cells if a 3-methoxy substituent is present at the salicylidene moiety. This study strengthened our intention to increase the cytotoxicity of [Fe^{III}(salophene)Cl] complexes by introduction of OCH_3 substituents (Scheme 1).

2. Results and discussion

2.1. Synthesis

The *N*,*N*′-bis(salicylidene)-1,2-phenylenediamine ligands were synthesized as previously described [9]. The commercially available 1,2-phenylenediamine was reacted in ethanol with two equivalents of the respective salicylaldehyde (Scheme 1). The precipitated products were collected and characterized by ^{1}H NMR spectroscopy (Fig. 1).

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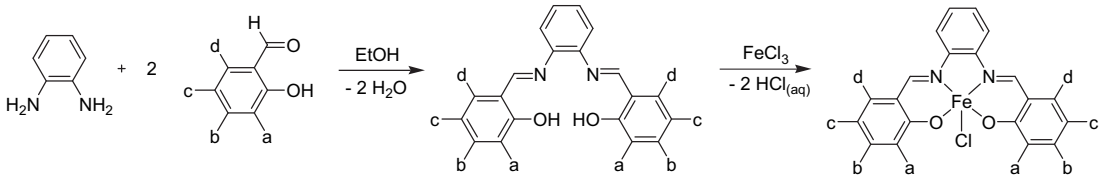

1,3,5,7,9 **2,4,6,8,10**

a	b	c	d	compound
H	H	H	H	**1**
H	H	H	H	**2**
OMe	H	H	H	**3**
OMe	H	H	H	**4**
H	OMe	H	H	**5**
H	OMe	H	H	**6**
H	H	OMe	H	**7**
H	H	OMe	H	**8**
H	H	H	OMe	**9**
H	H	H	OMe	**10**

Scheme 1. Synthesis pathway of the salophene ligands and their iron(III) complexes.

As shown in Fig. 1, the position of the methoxy group affected the OH signals ($\delta = 12-14$), while the resonance of the imine proton H^e was nearly identical for all investigated ligands. Methoxy groups located in ortho- or para-position (**3** and **7**) shifted the OH signal to higher field compared to that of the meta derivatives **5** and **9**. The most pronounced effect was observed for the para-substituted derivative **7**.

The observed shifts might be the consequence of H-bridges of different strength, due to the changed electron density at the oxygen. It is well known, that the phenolic protons of the salophene

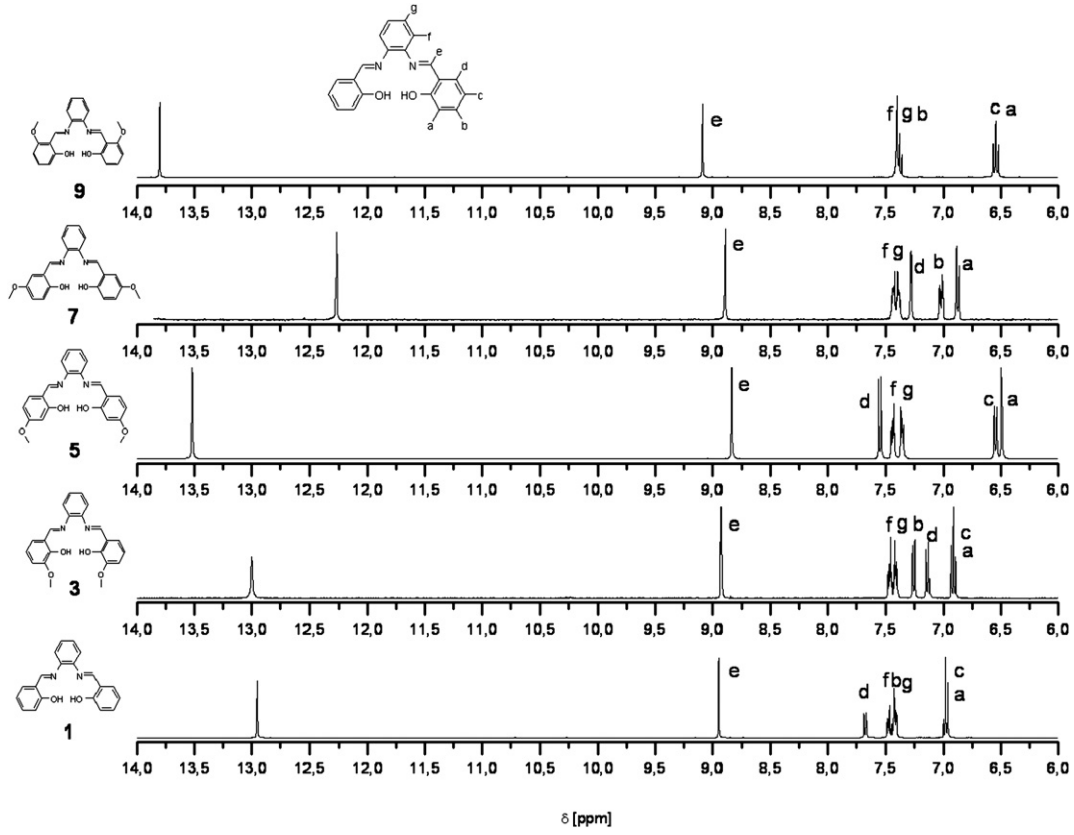

Fig. 1. ^{1}H NMR spectra of the ligands **1**, **3**, **5**, **7** and **9** in DMSO-d_6. The resonance peaks in the area of 12–14 ppm belong to the phenolic proton.

ligands were incorporated in H-bonds to the imine nitrogen O–H···N=C (salicylic effect) [11].

The resonance signals of the 1,2-phenylenediamine moiety (H_f and H_g, see Fig. 1 and Scheme 1) appeared in the same region irrespectively of OMe substituents located at the salicylidene, indicating no significant influence on the aromatic ring.

The paramagnetism of the iron complexes made a characterisation by NMR spectroscopy impossible. However, the coordination of the ligands to iron(III) could be verified by infra red and Raman spectroscopy (Table 1). The broad signal in the region from 3300 to 2400 cm^{-1} confirmed the involvement of the phenolic OH group and the imine nitrogen in H-bridges. Upon coordination, the band disappeared and the ν(C=N) at about 1610 cm^{-1} was shifted to lower frequencies. The $\Delta\nu$ of $\approx$10 cm^{-1} is in agreement with the literature. Two further vibration bands at about 1570 and 1470 cm^{-1} were influenced in the same way ($\Delta\nu$ = 30–40 cm^{-1}).

New bands arise in the region between 600 and 300 cm^{-1}. Vibration bands of the Fe–N ($\nu \approx 540$ cm^{-1}), the Fe–O ($\nu \approx 470$ cm^{-1}) and the Fe–Cl bond ($\nu \approx 320$ cm^{-1}) could be assigned by Raman spectroscopy (see Table 1) [12,13].

2.2. Solubility and stability in aqueous solution

Satisfying solubility and stability in aqueous solution are a prerequisite for successful in vitro studies. Testing at the limit of solubility and decomposition prior to cellular uptake would lead to inadequate results. Therefore, stock solutions of all complexes in DMSO (0.01 M) were diluted with aqua bidest. to 100 μM and stored at room temperature for 2 h. After filtration, the amount of iron was quantified by graphite furnace atomic absorption spectroscopy according to published procedures [14,15].

The solubility of the complexes depended on the position of the methoxy group: **2** (46.5 μM) < **4** (70.9 μM) < **6** (93.1 μM) < **8** (97.6 μM) < **10** (100.1 μM). However, in each case it is guaranteed that the complexes showed a higher solubility than the maximal concentration of 5 μM used in the in vitro tests.

Furthermore, the aqueous solutions were investigated for stability using HPLC. All complexes caused only a single peak in the HPLC chromatograms whose areas did not change during the observation time of 48 h.

It should be noted that the maximal amount of DMSO in the test solutions was 0.1%.

2.3. Biological activity

The biological activity was determined in MCF-7 and MDA-MB-231 breast cancer as well as in HT-29 colon carcinoma cell lines in relation to cisplatin. This drug is a well accepted reference for the design of metal based drugs.

Table 1
Selected Raman frequencies [cm^{-1}] of methoxy-substituted compounds **3**–**10**.

Compound	ν(C=N)	ν(Ar–O)	ν(Fe–N)	ν(Fe–O)	ν(Fe–Cl)
3	1612s	1573m 1467s	–	–	–
4	1602w	1546s 1435s	{529w}	{456m}	{340s}
5	1616m	1566w 1464w	–	–	–
6	1608w	1522w 1374w	{520w} {504s}	{480w} {439m}	{338s} {332s}
7	1623m	1571s 1489s	–	–	–
8	1616s	1535s 1463s	{636m}	{476w}	{321m}
9	1614m	1586s 1469s	–	–	–
10	1604w	1541s 1431s	{540w}	{480m}	{322s}

Cisplatin showed at a concentration of 5 μM cytostatic activity against MCF-7 and MDA-MB-231 cells (T/C_{corr} = 3%, respectively, see Fig. 2). On the HT-29 cell line it was less active (T/C_{corr} = 35%). The time response curves (5 μM) are characterized by a slow onset of activity and a maximum of cytotoxicity at the end of the experiment (150–200 h).

The lead structure of this study the [Fe^{III}(salophene)Cl] (**2**) was more active than cisplatin (compare Figs. 2 and 3). The highest activity was determined at the MDA-MB-231 cell line. Already at a concentration of 0.1 μM cytostatic effects were determined (min. T/C_{corr} = 10%). To achieve the same efficacy, 0.5 μM were necessary at the MCF-7 and 1 μM at the HT-29 cell line.

In contrast to cisplatin, **2** already reached its maximum inhibition of cell growth during an incubation time of 60 h followed by a recuperation of the cells. Such "V shaped profile" was documented by Bernhardt et al. during the establishment of this "crystal violet assay" [16]. As possible reasons for this behaviour they proposed inactivation of the drugs in the culture medium, the damage of only a subpopulation of cancer cells and the development of secondary resistance. In accordance with this, we determined the influence of the metal complexes on tumor cells over a time period up to 244 h and abstain from the determination of IC_{50} values, which documented the effects at only one time point.

For high cytotoxicity it was necessary to coordinate the ligands to iron. Both, $FeCl_3$ and free ligands did not significantly influence tumor cell growth within a concentration range of 1–5 μM (data not shown). The methoxy groups in the salicylidene rings led to a loss of tumor cell selectivity. The effects at MCF-7, MDA-MB-231 and HT-29 cells are comparable. Furthermore, only in the case of **8** a marginal recuperation of the cells was observed at the lowest concentration used (0.5 μM).

A 3-methoxy group reduced the antiproliferative effects. [Fe^{III}(salophene-3-OMe)Cl] (**4**) was inactive at 0.5 and 1 μM but reached the cytocidal area at 5 μM, indicating a steep concentration activity relation. The shift of the methoxy group from the 3- into the 4- (**6**) or the 6-position (**10**) enormously increased the growth inhibitory effects at the cell lines. Compounds **6** and **10** caused nearly identical curves as **4** but at 10 fold lower concentrations.

An exception regarding the concentration dependent response represents complex **8**. The activity of **8** was comparable to **4** at a concentration of 5 μM, but inhibited cell growth also at 1 and 0.5 μM.

2.4. Discussion

The graphs of the time response curves not only give information about possible development of resistance but also suggest about side effects observable in *in vivo* studies. Highly reactive platinum(II) complexes such as aquasulfatoplatinum(II) derivatives with very steep concentration activity curves were active in diverse mouse models but showed toxic side effects such as loss of body weight [17]. A reduction of the respective dose reduced both, the antitumor activity and the side effects nearly independent on the used tumor model. From the analysis of the graphs depicted in Fig. 4, a comparable behaviour can be deduced for **4**, **6**, and **10**.

The influence of methoxy groups on the cytotoxicity and the selectivity of iron(III)-salophene complexes is pronounced. Especially the loss of tumor cell selectivity was unusual and indicated a cell unspecific cellular interaction. Studies on the mode of action of this complex type were part of the first paper of this series [9]. [Fe^{III}(salophene)Cl] (**2**) was able to generate reactive oxygen species (ROS) and induced apoptosis but showed only low DNA binding. Although the iron(salophene) complexes possess a planar structure

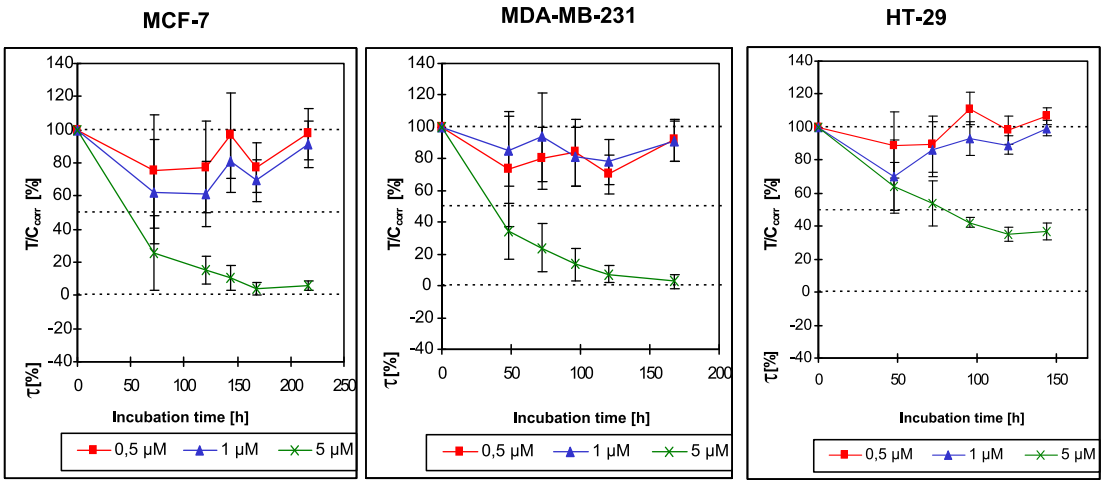

Fig. 2. Antiproliferative effects of cisplatin on MCF-7 and MDA-MB-231 breast cancer and HT-29 colon carcinoma cells.

comparable to very effective minor-groove binders CD-measurements and melting curve analysis provided no evidence of intercalation into the DNA double helix [9].

Exchanging the salophene by *N*,*N*′-bis(salicylidene)-1,2-cyclohexanediamine (saldach) reduced the in vitro effects drastically. An unequivocal mode of action cannot be deduced from these results, but it seems to be very likely that cell death is caused by interference with more than one intracellular target.

Methoxy groups at the salicylidene moiety seem to modify the pharmacological properties. It is obvious that the substitution in meta-position to the hydroxyl group bound to iron led to a much higher in vitro activity than in ortho- and para-position. Compared to [Fe^{III}(salophene)Cl] (**2**) this means an increase in activity at the MCF-7 and HT-29 cell lines and a slight loss of activity against MDA-MB-231 cells. It is very likely that methoxy substituents at **2** probably influence the cell unspecific redox behaviour of the complex. This is however speculative and has to be confirmed in a forthcoming study.

An indication is given by Routier et al. who showed that the para-substituted [bis(hydroxy)salen]iron(II) complex displayed cell-damaging properties because the hydroxyl group built a hydroquinone system cooperating with the redox-active metal to facilitate spontaneous formation of free radicals [6]. In our complexes such formation is impossible since the cleavage of the O-methyl ether was not observed under cell culture conditions. Nevertheless, the methoxy groups might influence the electron density at the Fe–O bond due to their electron donating effects. Interestingly the most favoured ortho- and para-position led to a reduction of activity.

This finding induced us to study the effects of +I/+M and −I/−M substituents at the salicylidene moiety on the cytotoxicity of the complexes. The results will be published in a forthcoming paper.

3. Materials and methods

3.1. General procedures

The following instrumentation was used: ^{1}H NMR, Bruker ADX 400 spectrometer at 400 MHz (internal standard, TMS); EI-MS spectra, CH-7A Varian (70 eV); IR spectra (KBr pellets), Perkin–Elmer model 580 A; FT-Raman spectra were measured on a Bruker RFS 100 spectrometer (excitation with 1064 nm line of Nd:YAG laser; power, 100 mW, resolution 3 cm^{-1}). Elemental C, H, N analyses were carried out with a Perkin–Elmer 240 B and 240 C elemental analyzer. Analyses indicated by the symbols of the elements or functions were within −0.4% of the theoretical values. Chemicals were obtained from Sigma Aldrich (Germany) and used without further purification. Compounds **1** and **2** were synthesized as recently published [9].

3.2. Synthesis of the ligands and their iron(III) complexes

Two equivalents of the respective salicylaldehyde (7.00 mmol) in ethanol (20 mL) were added dropwise to a solution of one

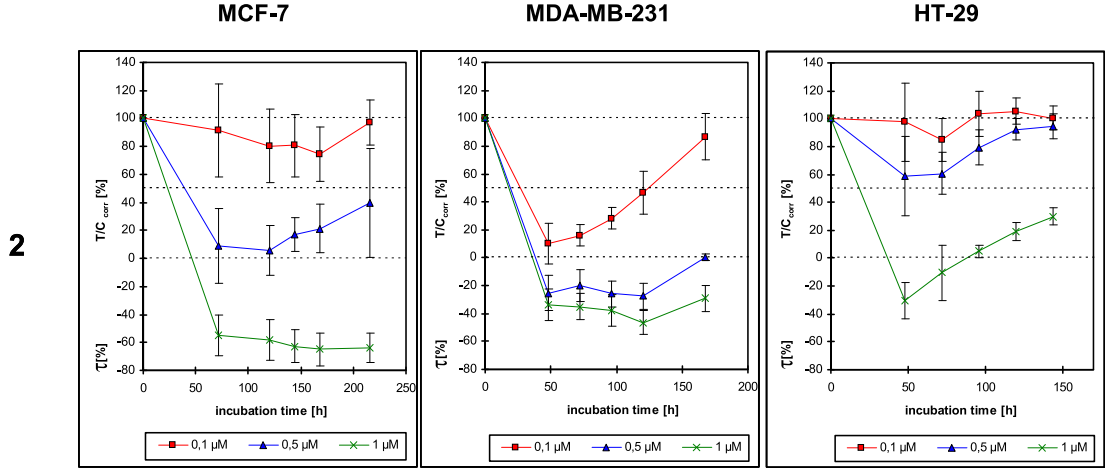

Fig. 3. Antiproliferative effects of [Fe^{III}(salophene)Cl] (**2**) on MCF-7 and MDA-MB-231 breast cancer and HT-29 colon carcinoma cells.

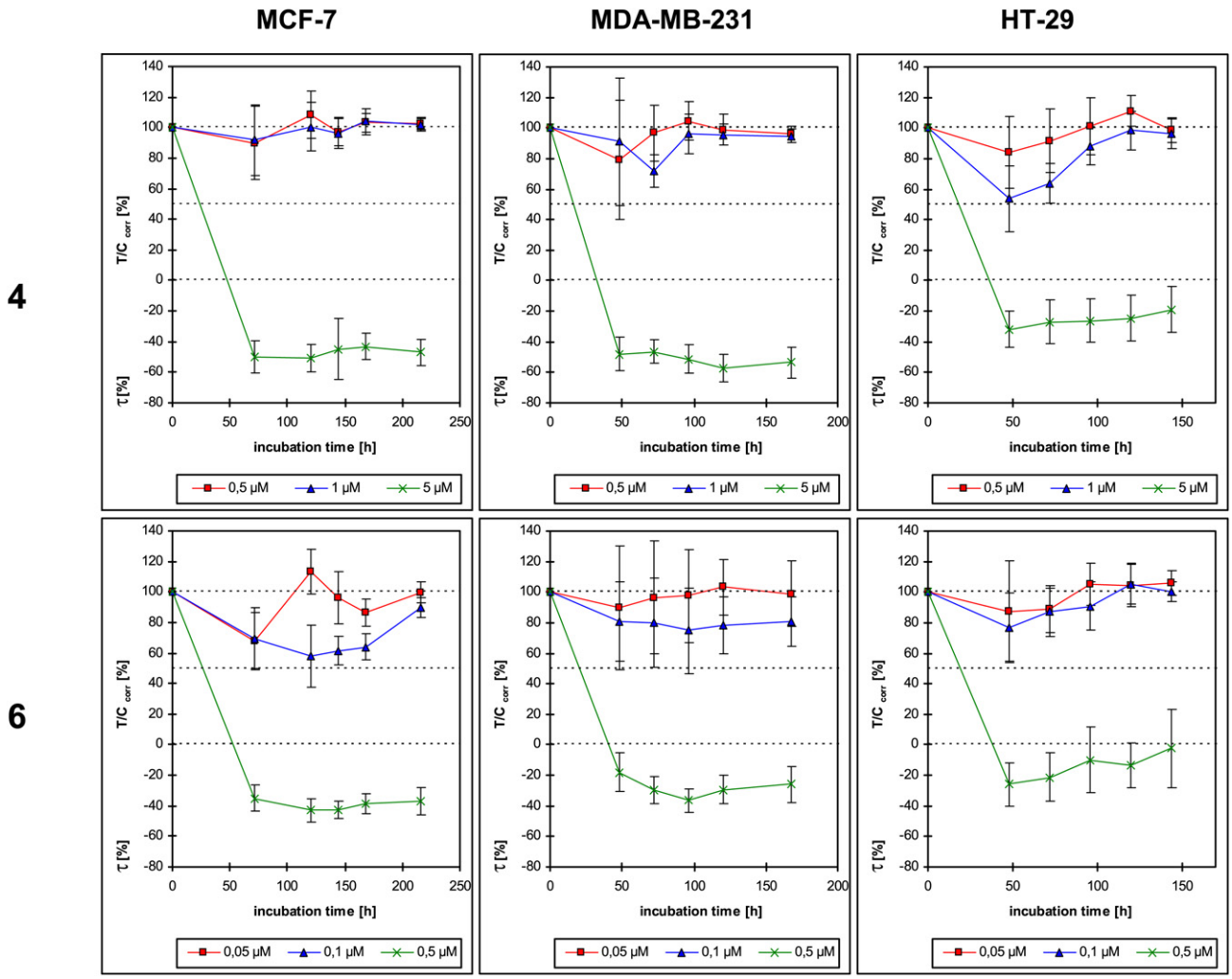

Fig. 4. Antiproliferative effects of the [Fe^{III}(salophene-OMe)Cl] complexes **4**, **6**, **8**, and **10** on the MCF-7 and MDA-MB-231 breast cancer and HT-29 colon carcinoma cells.

equivalent of 1,2-phenylenediamine in ethanol (10 mL). The mixture was stirred under reflux for 1–2 h and then allowed to cool down to room temperature. The product was collected, washed with ethanol and dried (P_2O_5). The obtained ligand (0.6 mmol) was dissolved in ethanol (10 mL) and heated to reflux in the presence one equivalent of $FeCl_3 \cdot 6H_2O$ in ethanol (5 mL). After 1–2 h, the mixture was cooled to room temperature, the solid was filtered off, washed with ethanol and dried in vacuo.

3.2.1. Synthesis of **3**

Compound **3** was obtained from 1,2-phenylenediamine and 3-methoxysalicylaldehyde. Yield: 1.31 g (3.48 mmol, 70%) of a crystalline, dark orange powder. ^{1}H NMR (DMSO-d_6): δ = 13.00 (s, 2H, Ar–OH), 8.93 (s, 2H, H_e), 7.48–7.40 (m, 4H, H_g + H_f, ^3J = 9.36 and 9.33 Hz, ^4J = 2.67 and 2.29 Hz), 7.26–7.24 (dd, 2H, H_b, ^3J = 7.83 Hz, ^4J = 1.16 Hz), 7.14–7.12 (dd, 2H, H_d ^3J = 8.08 ^4J = 1.09 Hz), 6.93–6.89 (m, 1H ^3J = 7.88 and 7.87 Hz), 3.81 (s, 3H, –OCH_3). IR (KBr): ν [cm^{-1}] = 3427 (br), 1612 (s), 1573 (m), 1466 (s), 1255 (s), 1205 (m). MS (EI, 90 °C): m/z (%) = 376 (74) [M^+]. Anal. $C_{22}H_{20}N_2O_4$ (C, H, N).

3.2.2. Synthesis of **4**

Compound **4** was obtained from *N*,*N*′-bis(3-methoxysalicylidene)-1,2-phenylenediamine (0.10 g, 0.27 mmol) and $FeCl_3 \cdot 6H_2O$ (0.072 g, 0.27 mmol). Yield: 72.10 mg (0.16 mmol, 58%) of a crystalline, black powder. IR (KBr): ν [cm^{-1}] = 1602 (w), 1579 (s), 1546 (s), 1446 (s), 1435 (s), 1313 (m), 1253 (s), 1199 (m), 529 (w), 456 (m), 340 (s). MS (EI, 200 °C): m/z (%) = 465 (30) [M^+], 430 (100) [M^+–Cl]. Anal. $C_{22}H_{18}N_2O_4FeCl$ (C, H, N).

3.2.3. Synthesis of **5**

Compound **5** was obtained from 1,2-phenylenediamine and 4-methoxysalicylaldehyde. Yield: 0.96 g (2.55 mmol, 84%) of a crystalline, yellow powder. ^{1}H NMR (DMSO-d_6): δ = 13.52 (s, 2H, Ar–OH), 8.84 (s, 2H, H_e), 7.56–7.54 (d, 2H, H_d, ^3J = 8.64 Hz), 7.45–7.34 (m, 4H, H_g + H_f, ^3J = 9.33 and 9.30 Hz, ^4J = 2.41 Hz), 6.56–6.53 (dd, 2H, H_c, ^3J = 8.62 Hz, ^4J = 2.37 Hz), 6.50–6.49 (d, 2H, H_a, ^4J = 2.31 Hz), 3.81 (s, 3H, –OCH_3). IR (KBr): ν [cm^{-1}] = 3433 (br), 1616 (m), 1566 (w), 1464 (w), 1513 (m), 1295 (m), 1204 (s). MS (EI, 120 °C): m/z (%) = 376 (52) [M^+]. Anal. $C_{22}H_{20}N_2O_4$ (C, H, N).

3.2.4. Synthesis of **6**

Compound **6** was obtained from *N*,*N*′-bis(4-methoxysalicylidene)-1,2-phenylenediamine (0.10 g, 0.32 mmol) and $FeCl_3 \cdot 6H_2O$ (0.07 g, 0.27 mmol). Yield: 104.0 mg (0.22 mmol, 84%) of a crystalline, black powder. IR (KBr): ν [cm^{-1}] = 1608 (w), 1574 (s), 1522 (w), 1374 (w), 1309 (s), 1266 (s), 1252 (s), 1208 (s), 520 (w), 504 (s), 480 (w), 439 (m), 338 (s), 332 (s). MS (EI, 275 °C): m/z (%) = 465 (70) [M^+], 430 (100) [M^+–Cl]. Anal. $C_{22}H_{18}N_2O_4FeCl$ (C, H, N).

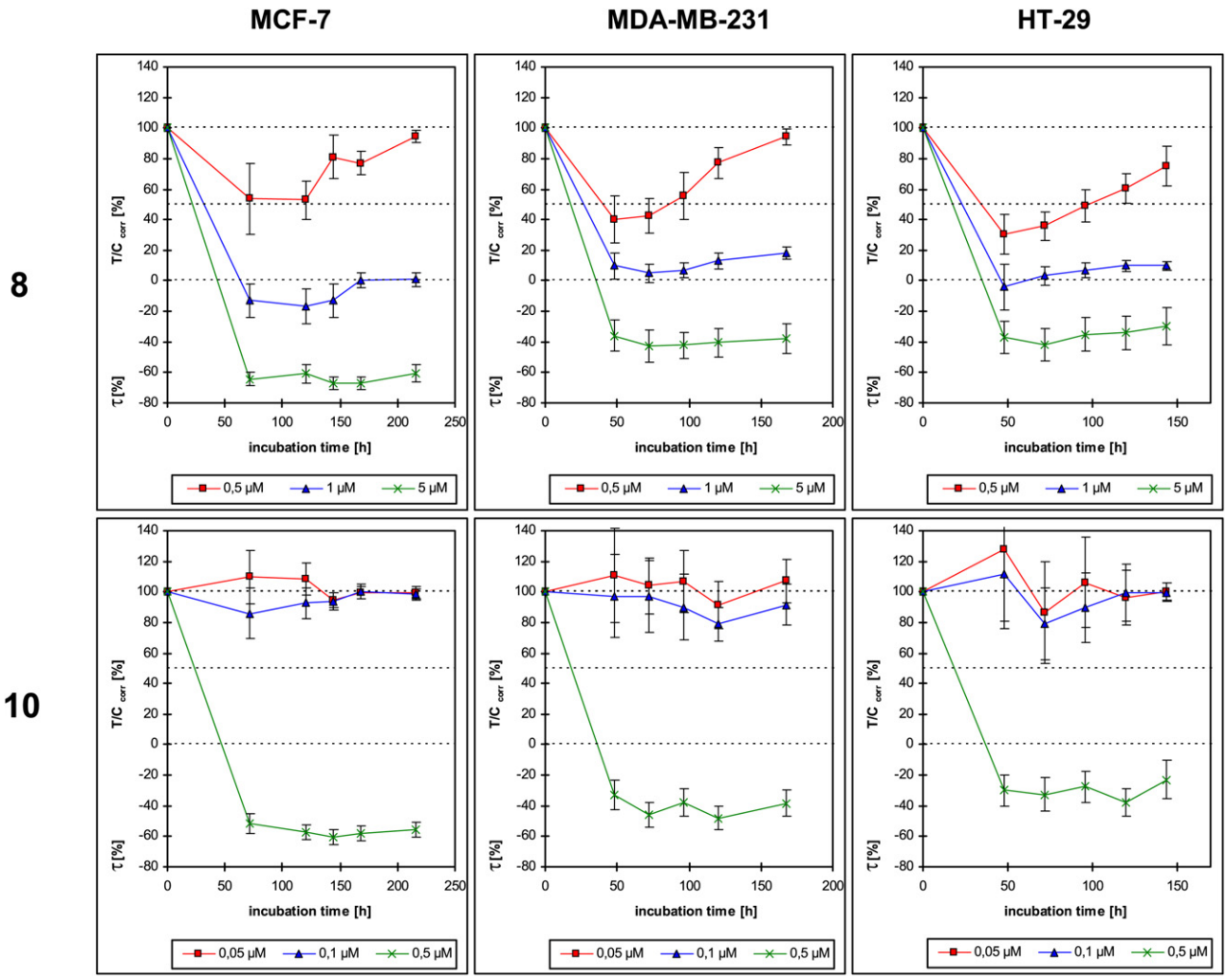

Fig. 4. (*continued*).

3.2.5. Synthesis of **7**

Compound **7** was obtained from 1,2-phenylenediamine and 5-methoxysalicylaldehyde. Yield: 1.19 g (3.16 mmol, 82%) of a crystalline orange powder. ^{1}H NMR (DMSO-d_6): δ = 12.27 (s, 2H, Ar–OH), 8.89 (s, 2H, H_e), 7.44–7.37 (m, 4H, H_g + H_f, ^3J = 9.34 and 9.35 Hz, ^4J = 2.63 and 2.64 Hz), 7.28–7.27 (d, 2H, H_d, ^4J = 3.09 Hz), 7.03–7.00 (dd, 2H, H_b, ^3J = 8.92 Hz, ^4J = 3.13 Hz), 6.89–6.86 (d, 2H, H_a, ^3J = 8.96 Hz). IR (KBr): ν [cm^{-1}] = 3419 (br), 1623 (m), 1571 (s), 1489 (s), 1270 (s), 1212 (m), 1160 (m). MS (EI, 150 °C): *m*/*z* (%) = 376 (63) [M$^+$]. Anal. $C_{22}H_{20}N_2O_4$ (C, H, N).

3.2.6. Synthesis of ***8***

Compound **8** was obtained from *N*,*N*′-bis(5-methoxysalicylidene)-1,2-phenylenediamine (0.14 g, 0.37 mmol) and $FeCl_3 \cdot 6H_2O$ (0.10 g, 0.38 mmol). Yield: 56.00 mg (0.12 mmol, 33%) of a crystalline, black powder. IR (KBr): ν [cm^{-1}] = 1616 (s), 1536 (s), 1469 (s), 1362 (m), 1287 (m), 1223 (m), 636 (m), 476 (w), 321 (s). MS (EI, 250 °C): *m*/*z* (%) = 465 (21) [M$^+$], 430 (100) [M$^+$–Cl]. Anal. $C_{22}H_{18}N_2O_4FeCl$ (C, H, N).

3.2.7. Synthesis of ***9***

Compound **9** was obtained from 1,2-phenylenediamine and 6-methoxysalicylaldehyde. Yield: 0.78 g (2.07 mmol, 63%) of a crystalline, light orange powder. ^{1}H NMR (DMSO-d_6): δ = 13.78 (s, 2H, Ar–OH), 9.08 (s, 2H, H_e), 7.41–7.31 (m, 6H, H_g + H_f, ^3J = 9.42 Hz, ^4J = 3.66 Hz, 2H, H_b, ^3J = 8.38 Hz, ^4J = 1.69 Hz), 6.55–6.51 (m, 4H, H_c + H_a, ^3J = 9.04 and 9.07 Hz), 3.84 (s, 3H, –OCH_3). IR (KBr): ν [cm^{-1}] = 3431 (br), 1614 (m), 1586 (s), 1469 (s), 1457 (s), 1358 (s), 1251 (s), 1187 (s), 1192 (s). MS (EI, 150 °C): *m*/*z* (%) = 376 (100) [M$^{+\cdot}$]. Anal. $C_{22}H_{20}N_2O_4$ (C, H, N).

3.2.8. Synthesis of ***10***

Compound **10** was obtained from *N*,*N*′-bis(6-methoxysalicylidene)-1,2-phenylenediamine (0.13 g, 0.34 mmol) and $FeCl_3 \cdot 6H_2O$ (0.097 g, 0.36 mmol). Yield: 97.00 mg (0.21 mmol, 60%) of a crystalline, black powder. IR (KBr): ν [cm^{-1}] = 1604 (w), 1572 (s), 1541 (s), 1459 (m), 1431 (s), 1366 (m), 1258 (m), 540 (w), 480 (m), 322 (s). MS (EI, 225 °C): *m*/*z* (%) = 465 (31) [M$^+$], 430 (100) [M$^+$–Cl]. Anal. $C_{22}H_{18}N_2O_4FeCl$ (C, H, N).

3.3. Biological methods

3.3.1. Cell culture conditions

The human MCF-7 and MDA-MB-231 breast cancer and HT-29 colon cancer cell lines were obtained from the American Type Culture Collection (ATCC). Both cell lines were maintained as a monolayer culture in L-glutamine containing Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose (PAA Laboratories GmbH, Austria), supplement with 10% fetal calf serum (FCS; Gibco, Germany) in a humidified atmosphere (5% CO_2) at 37 °C. The cell

lines were passaged twice a week after previous treatment with trypsin (0.05%)/ethylenediaminetetraacetic acid (0.02% EDTA; Boehringer, Germany).

3.3.2. In vitro chemosensitivity assay

The in vitro testing of the substances for antitumor activity in adherent growing cell lines was carried out on exponentially dividing human cancer cells according to a previously published microtiter assay [18]. Exponential cell growth was ensured during the whole time of incubation. Briefly, 100 μL of a cell suspension was placed in each well of a 96-well microtiter plate at 7200 cells/mL (MCF-7), at 3000 cells/mL (MDA-MB-231) and at 1600 cells/mL (HT-29) of culture medium and incubated at 37 °C in a humidified atmosphere (5% CO_2) for 3 d (MCF-7) and for 2 d (MDA-MB-231 and HT-29), respectively. Then the old medium was exchanged by 200 μL of fresh medium together with an adequate volume of a stock solution of metal complex to obtain the desired test concentration. Cisplatin was dissolved in dimethylformamide (DMF) while dimethylsulfoxide (DMSO) was used for all other compounds. Eight wells were used for each test concentration and the control containing the corresponding amount of DMF and DMSO, respectively. The medium was removed after appropriate incubation time. Subsequently, the cells were fixed with a solution of 1% (v/v) glutaric dialdehyde in phosphate buffered saline (PBS) and stored under PBS at 4 °C. Cell biomass was determined by means of a crystal violet staining technique as described earlier [16]. The effectiveness of the complexes is expressed as corrected T/C_{corr} [%] or τ[%] values according to the following equation:

$$\text{Cytostatic effect}: T/C_{corr}[\%] = [(T - C_0)/(C - C_0)] \times 100$$

$$\text{Cytocidal effect}: \tau[\%] = [(T - C_0)/C_0] \times 100$$

whereby T (test) and C (control) are the optical densities at 590 nm of crystal violet extract of the cells in the wells (i.e. the chromatin-bound crystal violet extracted with ethanol (70%) with C_0 corresponding to the density of the cell extract immediately before treatment. For the automatic estimation of the optical density of the crystal violet extract in the wells, a microplate autoreader (Flashscan S 12; Analytik Jena, Germany) was used.

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