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In vitro chemosensitivity of paclitaxel and other chemotherapeutic agents in malignant gestational trophoblastic neoplasms

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This is the first report on the ATP cell viability assay as a chemosensitivity test system for gestational trophoblastic neoplasms (GTN). We obtained chemosensitivity profiles in two established trophoblastic cell lines and four fresh tumors. Ten drugs were tested *in vitro* in the two cell lines JAR and JEG-3. The IC_{50} values of the 10 chemotherapeutic agents tested were very similar for both cell lines. The three most active drugs in these cell lines were VP-16, paclitaxel and vincristine. This is the first report on the activity of paclitaxel in trophoblastic cell lines. We furthermore evaluated this assay for chemosensitivity testing in four fresh malignant GTN tumors: one placental site trophoblastic tumor, one choriocarcinoma and two invasive moles. The placental site trophoblastic tumor specimen revealed to be rather chemoresistant *in vitro* whereas the other three tumors were chemosensitive. From our cell line data we conclude that the ATP cell viability assay is a practicable assay for chemosensitivity testing of GTN cell lines and gives repeatable results. However, the value of this assay for fresh GTN chemosensitivity testing needs to be defined.

Key words: ATP assay, chemosensitivity testing, gestational trophoblastic neoplasms, paclitaxel, placental site trophoblastic tumor.

Introduction

Several studies have shown the high curability of non-metastatic and 'low risk' metastatic gestational trophoblastic disease. These studies have shown virtually 100% cures of those patients.[1] For patients with 'high risk' gestational trophoblastic neoplasms (GTN), the achieved remission rates were considerably lower; however, this number has increased over the last years.[2] A multimodal therapy approach to this disease with the use of intensive combination chemotherapy, radiotherapy and occasional surgery, where indicated, has resulted in cure rates over 80% for patients with 'high risk' metastatic GTN.[3–6] The use of the WHO prognostic scores helped to identify a group of patients (score > 7) who are at the highest risk of recurrence and who need an intensive combination chemotherapy.[7,8] Factors responsible for treatment failures in patients with 'high risk' GTN are: presence of extensive disease at the time of diagnosis, lack of appropriate, aggressive initial treatment, and failure to respond to standard therapy.[5] Respectable salvage data have been reported for different regimens. The EMA-CO regimen is currently not only considered the regimen of choice in most 'high risk' patients, but also one of the most effective treatments for drug-resistant patients.[9,10] Since Newlands and Bagshawe have reported on the use of etoposide, several salvage regimens have been used containing VP-16 (etoposide):[9] VP-16, bleomycin, methotrexate;[11] VP-16, actinomycin-D, cisplatin;[12] and VP-16, bleomycin, cisplatin.[13] Another gestational tumor type called a 'placental site trophoblastic tumor' is thought to be resistant to chemotherapy. Resistance to chemotherapy is thought to correlate with the predominant cell type, which resembles the intermediate trophoblast. Although it is documented that some patients with placental site trophoblastic tumor may benefit from combination chemotherapy, surgical removal of the uterus continues to offer the best chance of long-term survival.[14]

In summary, for patients with 'high risk' metastatic disease, for patients with recurrent disease and for patients with placental site trophoblastic tumor there is a need for more active drugs that can be given in combination with other cytotoxic agents

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such as VP-16. The low incidence of malignant GTN makes testing of new chemotherapeutic agents extremely difficult. Furthermore, there are currently only few *in vitro* test systems established that allow new drugs to be tested for choriocarcinoma in cell lines and fresh tumor.[15] We explored the usefulness of the ATP assay for testing drugs for malignant GTN. A panel of 10 drugs, including paclitaxel, was tested in two established malignant trophoblastic cell lines that were available from the American Type Culture Collection (ATCC). Furthermore, we also started to test fresh trophoblastic tumors with the most active drugs found in cell line experiments: paclitaxel, VP-16 and vincristine. Chemosensitivity testing of a placental site trophoblastic tumor, a choriocarcinoma and two invasive moles is presented.

Materials and methods

Cell lines

The human trophoblastic cell lines JAG and JEG-3 were obtained from the ATCC: JAR is derived from an untreated, malignant trophoblastic tumor of the placenta. It produces estrogen, progesterone, gonadotropin and lactogen in culture. A culture at passage 717 was obtained. This line parallels in hormone function with the high yields of multiple human hormones produced by the BEWo line.[16,17] JEG-3 is one of six clonally derived lines isolated by Kohler *et al.* in 1971.[18] Therefore, fragments of the Wood's strain of the Erwin–Turner tumor in its 387th passage in the hamster cheek pouch were used to set up explant cultures. Colonies were isolated and recloned after propagation. JEG-3 released human chorionic gonadotropin, human chorionic somatomammotrophin and progesterone. In the nude mouse model it forms malignant tumor consistent with choriocarcinoma.[18]

JAR was maintained with RPMI, JEG-3 with Eagle's MEM (Gibco®, USA/Switzerland). Medium was prepared with 10% fetal bovine serum. Cells were incubated at 37°C with 5% CO_2. Medium was replaced three times weekly and cells were subcultured weekly following detachment with trypsin–EDTA as described elsewhere.[15]

Drugs

The following drugs were tested using the reported plasma peak concentrations (PPC) as reference values:[19–21] doxorubicin (Farmitalia) 0.5 μg/ml, paclitaxel (Bristol-Myers Squibb) 4.27 μg/ml, pirarubicin (Behring, Germany) 0.5 μg/ml, methotrexate (Lederle) 2.8 μg/ml, actinomycin-D (MSD) 0.1 μg/ml, vincristine (Eli Lilly) 0.4 μg/ml, VP-16 (Bristol-Myers Squibb) 15 μg/ml, carboplatin (Bristol-Myers Squibb) 5 μg/ml, cisplatin (Bristol-Myers Squibb) 2.5 μg/ml. For the active metabolite of cyclophosphamide, 4-hydroxy-cyclophosphamide (4-HC), a concentration of 6.0 μg/ml was used. This is 20% of the PPC of cyclophosphamide and reflects the resultant plasma level of this metabolite after intrahepatic conversion.[19,22] The drugs were tested at 0.1, 0.2, 0.5, 1.0 and 5.0, PPC. Since these relatively high concentrations of VP-16 and taxol resulted in almost complete cell kill at 0.1 PPC, experiments were also done with 0.01, 0.02, 0.05, 0.1 and 0.5 PPC, and 0.0001, 0.001, 0.002, 0.005, 0.01 and 0.05 PPC. These results were used to calculate the IC_{50} values after the median effect principle.[23]

ATP cell viability assay

The ATP cell viability assay in cell lines was performed as described in detail elsewhere.[15,24,25] Briefly, triplicate wells were used for each agent tested using 24-well tissue culture plates. Twenty thousand cells per well were plated 24 h preceding a 90 min exposure to each drug. Each experiment was repeated at least three times. Dose–response curves were obtained by comparing intracellular ATP with untreated controls on day 6. ATP was extracted from the cells *in situ* with 4% trichloro-acetic acid. ATP luminescence was determined with the luciferin–luciferase reaction as previously described.[25] In this study, percent of control ATP was defined as the survival fraction. For fresh tumor testing we used the same assay system with minor changes, such as an anchorage-independent culture system, different media and different lysing agents, as described earlier by Koechli *et al.*[15,24,26]

Criteria of response

Dose–response curves of JAR and JEG-3 were determined for 10 drugs. A drug-induced reduction of control ATP level of at least 50% at 0.5 PPC was defined as a sensitive response, while a less than 50% reduction of cellular ATP was considered resistant, as previously described.[27] In fresh tumor, sensitivity was defined as 70% or more reduction in ATP concentration versus control, partial sensi-

tivity 50–69% ATP reduction and resistance 0–49% ATP reduction at 25% of the PPC as previously defined by Koechli in 1994[15,24] and Sevin *et al.* in 1988.[25]

Data analysis

The IC_{50} values for all tested drugs were calculated, using the median effect plot of log (f_a/f_u) versus log C described by Chou *et al.*[23,28] (f_a = fraction affected, f_u = fraction unaffected, C = drug concentration).

Fresh tumor specimen

The placental site trophoblastic tumor came from a 26 year old patient. The preoperative β-HCG was 200 IU/l. The tumor was obtained from the uterus after hysterectomy. The choriocarcinoma came from a 20 year old female with metastatic disease. The cells were obtained from a pleural effusion. The pretherapeutic β-HCG was 39 000 IU/l. The two invasive moles were obtained from 32 and 39 year old patients. Both had hysterectomies. The specimens were mechanically–enzymatically disaggregated, cultured for 6 days and analyzed with the same ATP assay as previously described.[15,24] We demonstrated earlier that in fresh tumor the 0.25 PPC is suitable to predict clinical outcome.[15,24]

Results

We were able to obtain dose–response curves for all drugs tested and to calculate the IC_{50} values for each drug. Figure 1 shows the dose–response curves in JAR for six commonly used drugs in the treatment of gestational trophoblastic disease. On the y-axis, the percent of control ATP representing the survival fractions is demonstrated. On the x-axis, the administered dose of the drug is shown in PPC. For actinomycin-D we found a very flat dose response in this cell line. Even at the highest dose of 5 PPC there was almost no cell kill. For the other five drugs tested we found a strong dose–response relationship (Figure 1). However, only etoposide, methotrexate and vincristine showed a cell kill of more than 50% at 0.5 PPC. 4-HC and cisplatin led to a high cell kill only at a high doses of 1 PPC and more. In Figure 2 the effects of the less commonly used drugs such as paclitaxel, pirarubicin, doxorubicin and carboplatin are demonstrated. We found a very

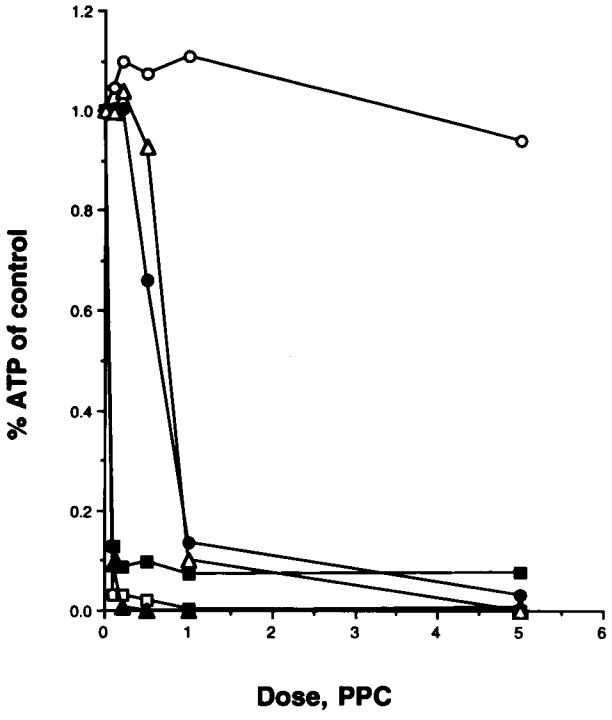

Figure 1. Dose–response curves in JAR for six chemotherapeutic agents that are often given for malignant trophoblastic neoplasms: actinomycin-D (○), cisplatin (●), etoposide (□), methotrexate (■), vincristine (▲) and 4-HC (△). The x-axis shows the dose in PPC and the y-axis shows the percent ATP of control.

good dose response for pirarubicin and paclitaxel, and a weak dose response for carboplatin and doxorubicin. Since the given concentrations of paclitaxel and VP-16 resulted in an almost complete cell kill at 0.1 PPC, experiments were also done with lower concentrations as described above. The dose–response curves for these two drugs at very low concentrations in JAR are shown in Figure 3. The dose–response curves in JEG-3 for the same six commonly used drugs as in JAR were similar to the curves found in JAR-3 (curve not shown). Again, for actinomycin-D we found a very flat dose–response curve. For cisplatin a strong cell kill could only be obtained when a high drug concentration of 5 PPC was given. The dose–response curve of 4-hydroperoxy-cyclophosphamide in JEG-3 was very similar to that obtained in JAR. As demonstrated in JAR, doxorubicin and carboplatin were much less active than pirarubicin and paclitaxel. When lower concentrations of paclitaxel and VP-16 were given in JEG-3, a better dose–response curve was obtained (Figure 3). The calculated IC_{50} values for both cell lines for these 10 drugs are summarized in Table 1. The most active drugs were VP-16, paclitaxel and

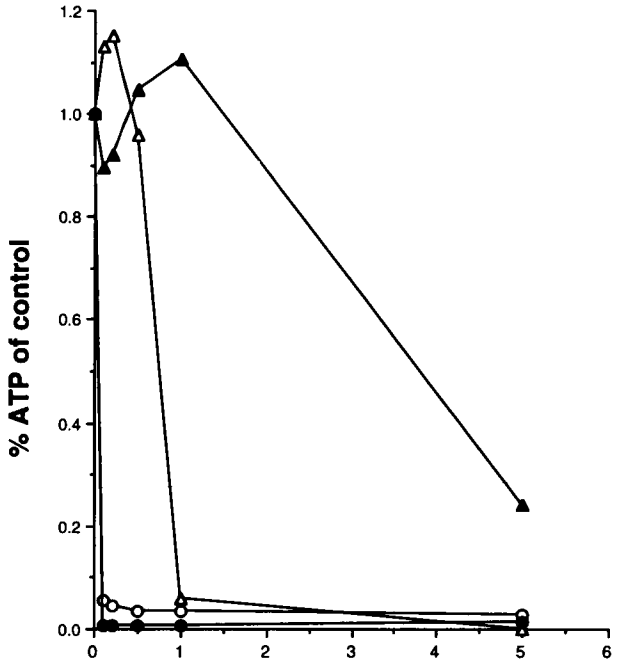

Figure 2. Dose–response curves in JAR for four chemotherapeutic agents that are rarely given for malignant trophoblastic neoplasms: doxorubicin (▲), carboplatin (△), pirarubicin (○) and paclitaxel (●). The *x*-axis shows the dose in PPC and the *y*-axis shows the percent ATP of control.

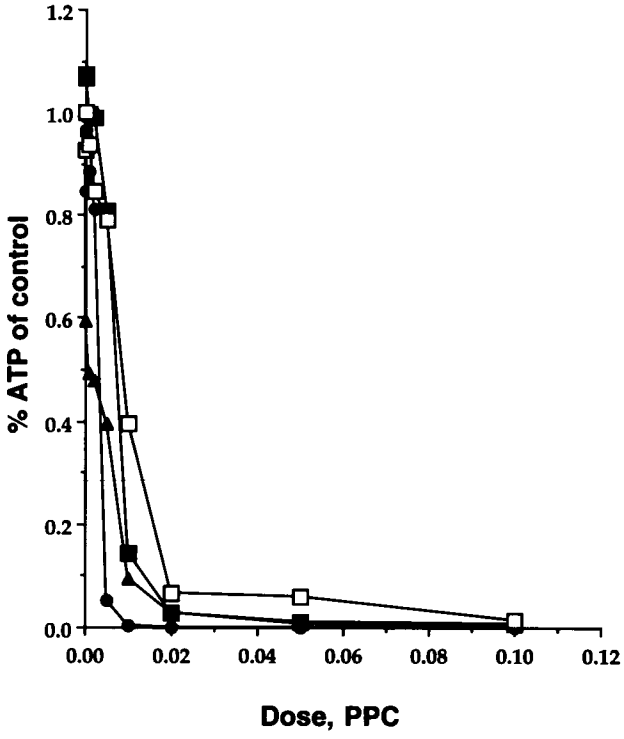

Figure 3. Dose–response curves in JAR and in JEG-3 for two *in vitro* very active drugs in malignant trophoblastic neoplasms: paclitaxel (● in JAR; ■ in JEG-3) and VP-16 (▲ in JAR; □ in JEG-3). The *x*-axis shows the dose in PPC and the *y*-axis shows the percent ATP of control.

Table 1. IC_{50} values[a] of 10 chemotherapeutic agents tested in JAR and JEG-3[b]

	JAR		JEG- 3	
	mean[c]	SD[d]	mean[c]	SD[d]
VP-16	0.0013	0.0001	0.0015	0.0015
TAX	0.0015	0.0002	0.0025	0.0003
VIN	0.0120	0.002	0.0270	0.002
PIRA	0.0420	0.006	0.0414	0.003
MTX	0.0800	0.01	0.0485	0.008
4-HC	0.73	0.06	0.30	0.06
CARBO	1.29	0.11	1.37	0.09
DDP	1.44	0.2	1.012	0.18
ADR	4.27	0.3	1.075	0.11
ACT-D	35.74	4.0	25.75	2.62

[a]Inhibition concentration at 50% cell kill.
[b]Values in PPC.
[c]Mean of three experiments.
[d]± standard deviation.

vincristine, with very low IC_{50} values. Furthermore, we found that the new anthracycline analog pirarubicin is also an active drug in these cell lines when compared with doxorubicin. The drugs 4-HC, carboplatin, cisplatin, doxorubicin and actinomycin-D had poor activity in these two cell lines.

The chemosensitivity profiles for the fresh cultured malignant trophoblastic tumor specimens are

shown in Figure 4. We evaluated in fresh tumor two combinations of the three most active drugs found in cell line experiments. For each tumor the dose–response curves for paclitaxel plus VP-16 and VCR plus VP-16 could be calculated. The invasive moles and the choriocarcinoma showed a very strong dose response. At 0.25 PPC all three tumors had survival fractions $\leq$ 30%. This emphasizes the relative chemosensitivity of these primary malignant GTN tumors–a fact that is clinically known.[9] However, the placental site trophoblastic tumor was resistant to the combination of paclitaxel and VP-16 and showed only a partial sensitivity to the combination of vincristine plus VP-16. At 4.0 PPC all tumors showed an almost hundred percent cell kill with the exception of vincristine plus VP-16 in the placental site trophoblastic tumor. For this combination a very flat dose–response curve could be observed in this rare disease.

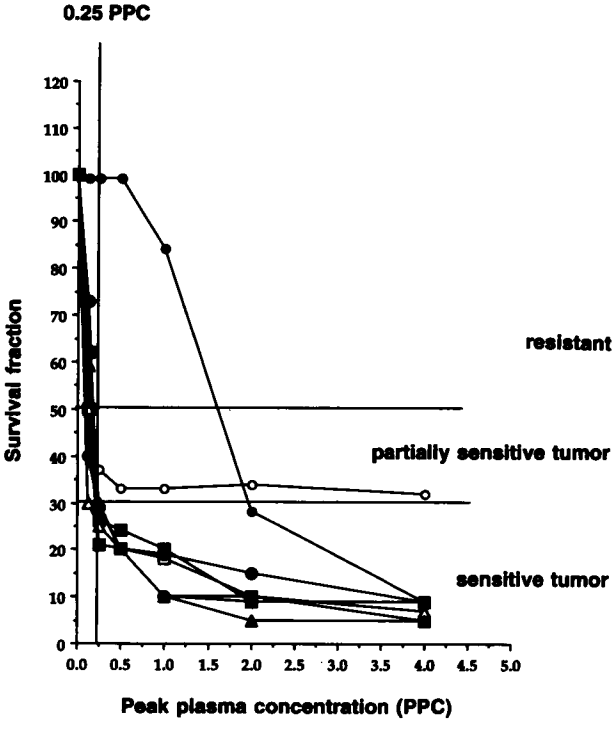

Figure 4. Dose–response curves for paclitaxel (Tax) plus VP-16 and vincristine (VCR) plus VP-16 in a placental site trophoblastic tumor (A), a choriocarcinoma (B) and two invasive moles (C and D). The x-axis shows the dose in PPC and the y-axis shows the survival fractions. ●, Tax- VP-16 A; ○, VCR-VP-16 A; □, Tax-VP-16 B; ■, VCR-VP-16 B; △, Tax-VP-16 C; ▲, VCR-VP-16 C; ●, Tax-VP-16 D; ●, VCR-VP-16 D.

Discussion

This is a report on the feasibility of the ATP assay as a chemosensitivity test system for GTN. We obtained chemosensitivity profiles in two established trophoblastic cell lines, two invasive moles, a choriocarcinoma, and in one placental site trophoblastic tumor, which is known to be relatively resistant to chemotherapeutic agents. Ten drugs were tested *in vitro* in two cell lines. Six drugs were commonly used drugs in the treatment of GTN and four drugs were new or seldom used for this disease. JAR is a cell line that is derived from an untreated, malignant trophoblastic tumor of the placenta and JEG-3 is one of the six clonally derived lines isolated by Kohler *et al.*[18] This cell line is known to form malignant tumor consistent with choriocarcinoma in the nude mouse model.[18] These two cell lines are therefore suitable to test chemotherapeutic agents that are considered to be active against GTN. Since it is seldom that these tumors are clinically diagnosed, we investigated whether the ATP assay could be used to evaluate drug effects in GTN cell lines as well as in fresh tumor. As shown in Table 1, the IC_{50} values of the 10 chemotherapeutic agents tested in JAR and in JEG-3 were very similar for both cell lines. The three most active drugs in both cell lines were VP-16, paclitaxel and vincristine (Table 1 and Figures 1–3). Pirarubicin, a new anthracycline analog, was significantly more active than doxorubicin. The two drugs actinomycin-D and methotrexate, which are given clinically as first line therapy in non-metastatic GTN, showed a different dose response. Actinomycin-D was resistant in both cell lines, whereas methotrexate showed sensitivity (Figure 1). These two cell lines can therefore also be considered as a test system for actinomycin-D resistant tumors. In both cell lines the platin analogs cisplatin and carboplatin showed resistance as well as the active metabolite of cyclophosphamide. As shown in Figure 3, drug concentrations of VP-16 and pac-litaxel in the range of 0.002–0.02 PPC gave the best dose–response curves. VP-16 and paclitaxel were both slightly less active in JEG-3 than in JAR. In summary, five of the 10 drugs tested were resistant in both cell lines. The active chemotherapeutic agents were: VP-16, paclitaxel, vincristine, pirarubicin and methotrexate. The corresponding IC_{50} values for these five drugs in the two cell lines JAR and JEG-3 ranged from 0.0013 to 0.08 PPC. This is the first report on the activity of paclitaxel in trophoblastic cell lines. Further testing of this agent as a single drug and in combination seems justified not only in cell lines but also in fresh tumor specimens. The relatively low standard de-

viations supported the already known low inter-assay variability (Table 1).[15,24]

One of the advantages of the ATP cell viability assay is that the same test system, with only minor changes, can be used for both cell line and for fresh tumor experiments.[15,24] This has been shown for ovarian and breast cancer.[15,24,25] To show the feasibility in GTN, we presented chemosensitivity profiles of four fresh malignant GTN tumors. The placental site trophoblastic tumor is rare and thought to be chemoresistant.[30,31] As shown in Figure 4, we were able to confirm that this tumor is relatively drug resistant. Only the combination VP-16 plus vincristine was partially sensitive. The three other tumors were all chemosensitive to the tested two drug combinations and a strong dose response for all combinations could be observed. Nevertheless we found significant heterogeneity in the dose response for the tumors tested. Heterogeneity of chemosensitivity in fresh tumors is well known. This indicates that individual chemosensitivity testing is important. The *in vitro/in vivo* correlations of the *in vitro* chemosensitivity results with the ATP assay from other tumor types demonstrated that this assay predicts both sensitivity and resistance in 86%.[32]

In this study we could show that the ATP cell viability assay gives repeatable dose–response curves in GTN cell lines. With the testing of the four malignant trophoblastic tumors we could also demonstrate that chemosensitivity testing with the ATP cell viability assay is feasible in this disease. However, more experience with fresh tumor testing of this rare disease is necessary to define the exact value of this assay. Especially in resistant tumors we consider chemosensitivity testing important to aid drug selection. However, there are also some limitations. In GTN it is not always easy to obtain tumor tissue for testing since many patients are not treated by surgery. Furthermore, there are some other limitations such as altered pharmacological parameters *in vitro*, reduced blood supply in hypoxic tumors *in vivo* and *in vivo* acquired drug resistance after repeated treatments with cytotoxic drugs.[33–35] These factors should be kept in mind in future investigations.

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