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Original Article

The potential of brentuximab vedotin, alone or in combination with current clinical therapies, in the treatment of testicular germ cell tumors

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Abstract: Testicular germ cell tumors (TGCTs) are the commonest tumors in young men. With the advancement of chemotherapies, most TGCTs are successfully cured, even when diagnosed at an advanced and metastatic stage. However, a proportion of often young patients, median age 35-40, with advanced disease are not cured and will inevitably die. Therefore, there is an unmet need in this small population of young patients who are candidates for experimental approaches. We investigated a new therapeutic option for this group of patients, aiming to significantly improve their outcome. In recent years, many targeted therapies have been developed which demonstrated high efficacy and low toxicity. Brentuximab vedotin, a monomethyl auristatin E conjugated CD30 antibody, targets CD30 to kill cancer cells. As a large proportion of TGCTs express CD30, in particular embryonal carcinomas, we investigated *in vitro* the efficacy of brentuximab vedotin in treating TGCTs as a single therapy and in combination with commonly used chemotherapy drugs. We determined CD30 expression levels in 12 TGCT cell lines, including three cisplatin resistant sublines. In general, the efficiency of cancer cell inhibition by brentuximab vedotin correlates with CD30 expression, but there were some exceptions. We also determined the efficacy of brentuximab vedotin in combination with commonly used chemotherapy drugs and found synergistic/additive effects with etoposide, paclitaxel and SN-38. However, cisplatin, the most commonly used chemotherapy drug in TGCT treatment, exhibited antagonism and we showed that cisplatin selectively kills CD30 positive cells. We also found that certain agents, which have been reported to induce CD30 expression in other human malignant diseases, including DNA demethylation drugs, methotrexate and CD30 ligands, were unable to enhance CD30 expression or brentuximab vedotin efficacy in TGCT cells. This study will help to design clinical trials using brentuximab vedotin for the treatment of TGCTs, either as a single agent or in combination with current clinical therapies.

Keywords: Testicular germ cell tumor, CD30 expression, brentuximab vedotin, chemotherapy, cisplatin, drug combination

Introduction

Testicular germ cell tumors (TGCTs) are the commonest tumors in young men. There are two broad types of TGCTs, seminoma and non-seminoma. In the current management of locally advanced and metastatic cases, chemotherapy has a central role. The use of cisplatin, most commonly with etoposide and either bleomycin or ifosfamide, cures the majority of patients. Those with very high levels of tumor markers or with liver, brain or bone metastases are less likely to be cured. Patients whose tumors are refractory to primary therapy have a cure rate between 25-50%. This is normally achieved by further chemotherapy using cisplatin, or high dose of carboplatin based chemotherapy in combination with surgery. Less fit patients are much less likely to be cured by these approaches. In addition, following the failure of an initial salvage therapy, the cure rate is under 25% [1-4]. These TGCT patients are often young (median age 35-40), thus novel therapeutic approaches need to be developed urgently.

Monoclonal antibody therapy directed against unique antigens expressed on cancer cells has been successfully applied in many cancer treatments and antibody-drug conjugates (ADC) have extended this technology, allowing delivery of high doses of cytotoxic drugs to cancer cells while largely sparing normal tissues [5]. Brentuximab vedotin (Adcetris) is a CD30-directed monoclonal antibody conjugated to the potent inhibitor of microtubule polymerization, monomethyl auristatin E. It not only selectively targets and kills CD30-expressing cancer cells, but also kills surrounding CD30 negative cancer cells through a bystander effect [6-8]. Preclinical study of brentuximab vedotin in Hodgkin lymphoma and anaplastic large cell lymphoma showed excellent therapeutic effect [5]. Following further clinical trials, brentuximab vedotin has proven to be a highly promising drug for patients with CD30-positive lymphomas with limited side-effects. It has been approved for the treatment of advanced Hodgkin lymphoma and anaplastic large cell lymphoma [5].

CD30 is expressed by most embryonal carcinomas and yolk sac tumors, the aggressive forms of non-seminomous TGCTs [9-15]. In seminomas, previous reports vary and generally around 10-20% of them were positive [9, 10, 12, 13, 16]. Our and other's previous studies in clinical samples have shown that chemotherapy can change CD30 expression from positive to negative in more than half of the embryonal carcinoma cases [11, 15]. It has been reported that TGCTs with retained CD30 expression after intensive treatment were associated with a significantly poorer prognosis [14], which suggests that CD30 is a valid treatment target. Two recent clinical studies, each with a few patients, have demonstrated the potential benefit of treating cisplatin-resistant GCTs with brentuximab [17, 18]. As CD30 expression in TGCTs decreases following chemotherapy, it is possible that brentuximab vedotin may be more efficient in treating TGCT if used before chemotherapy with certain drugs.

Since TGCTs are commonly treated with platinum based regimens, in this study we investigated, using *in vitro* testicular embryonal carcinomas cell line models, the influence of cisplatin on CD30 expression levels and the sensitivity to brentuximab vedotin. As there is limited data on the combination of brentuximab vedotin with chemotherapy drugs, we also determined which chemotherapy drugs commonly used for TGCT treatment may have synergistic or additive therapeutic effect with brentuximab vedotin. CD30 expression in a large number of post-radiotherapy non-seminomatous TGCT cases were also investigated.

Materials and methods

Patient tissue samples

Post-radiotherapy TGCT tissue blocks (1969-1983) were retrieved from St Bartholomew's Hospital, Barts Health NHS, London, UK, and reviewed (DB) for remaining TGCT lesions to construct tissue microarrays as previously described [19]. 91 cases were included in this study and the use of patient samples was approved by the National Research Ethics Service committee, London City & East with a Research Ethics Committee reference of 09/H0704.

Cell lines

Non-seminomatous TGCT cell lines 833K parental cisplatin sensitive, 833K cisplatin resistant subline (833KR), Susa parental cisplatin sensitive, Susa cisplatin resistant subline (SusaR), GCT27 parental cisplatin sensitive, GCT27 cisplatin resistant subline (GCT27R), GCT44, TERA-1, NTERA-2, NCG2102 and 577MF and a seminoma cell line TCam-2 were used. The cisplatin resistant lines were established by the repeated passaging of cells through media containing low doses of cisplatin [20]. The prostate cancer cell lines PC3, 22Rv1, DU145, LNCaP and osteosarcoma cell line MG63 were also used. Cells were maintained in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin in a controlled atmosphere with 5% CO_2 at 37°C, except for TCam-2 cells which was maintained in RPMI 1640 (Gibco) instead of Dulbecco's Modified Eagle Medium.

Drugs used for TGCT treatment

Brentuximab vedotin was kindly provided by MILLENNIUM PHARMACEUTICALS, INC free of charge through a research collaboration agreement. The chemotherapy drugs used are cisplatin (TEVA UK Ltd), methotrexate (Sigma), etopo-

side (Sigma), SN-38 (Sigma), paclitaxel (Sigma) and actinomycin-D (Sigma).

Cell treatment with intention to manipulate the expression of CD30

Gene knockdown by siRNA was performed as previously described [21] using the CD30 siRNA from Dharmacon. CD30 Ligand/TNFSF8 (R&D systems) at a concentration of 50 ng/ml was cross-linked using 5 μg/ml His Tag monoclonal mouse antibody Clone AD1.1.10 (R&D systems, MAB0500) before being used for cell treatment. Cells were also treated with cisplatin at IC50 concentrations of relevant cell lines (1.5 μM for 833K and 2.5 uM for GCT27), 10 μM methotrexate and DNA demethylation agents 5-Aza-2'-deoxycytidine (Sigma) at the concentration of 5 μm and cladribine (Sigma) at the concentration of 3.5 μM for 72 hours to determine CD30 expression changes. All these experiments were done in six well cell culture plates.

Assessment of cell response to drugs

Cell response to drugs was assessed by measuring cell viability using the CellTiter 96® AQueous assay (Promega) as previously described [21, 22]. Briefly, cells were seeded in 96-well plates and after 24 hours cells were treated with serial dilutions of drug dosages. Cell viability was assessed after 72 hours of treatment and dose response curves were generated based on relative cell viability normalized to untreated controls.

Quantitation of synergism and antagonism in drug combinations

Combined drug treatment were performed as previously described [22] with different combinations of IC30 and IC50 between two drugs. Killing effect values were used to examine synergism and antagonism of drugs using CompuSyn software (http://www.combosyn.com/register.html). Combination index (CI) > 1 indicates antagonistic effect, CI = 1 indicates additive effect and CI < 1 indicates synergism [23].

Western blot analysis

Western blotting was performed as previously described [24] using rabbit monoclonal anti-CD30 EPR4102 (Abcam ab134080), and anti-β-actin (A5441, Sigma) antibodies. Briefly, cells were lysed in RIPA buffer and protein concentration was determined by Bradford assay. Proteins were mixed with NuPAGE® LDS Sample Buffer (BioRad) and reducing agent (BioRad) and denatured at 90°C for 5 min. Protein samples were loaded on 10% polyacrylamide gels and transferred onto PVDF membranes (Millipore, UK) after separation. Membranes were blocked with 5% skimmed powder milk in Tris Buffer Saline/0.1% Tween-20 and incubated overnight with primary antibodies, followed by secondary peroxidase conjugated antibodies (Fisher Scientific) for 1 hour at room temperature. Protein detection was performed with Immobilon Western Chemiluminescent HRP Substrate (Millipore). β-actin was used as loading control. Relative expression of CD30 was quantified in ImageJ.

Immunocytochemistry analysis

Cells were cultured on glass cover slips and then fixed with 10% formalin for 20 minutes, followed by PBS wash and permeabilization in 0.5% Tween 20 in PBS. Primary rabbit monoclonal anti-CD30 antibody diluted 1:2000 in PBS with 1% BSA was applied for 1 hour at room temperature. Primary antibody was omitted and only 1% BSA in PBS diluent was used for negative control. After wash with 1% Tween 20 in PBS (PBST), secondary biotinylated antibody from the Vectastain Universal Elite ABC kit (Vector Lab) was applied for 30 minutes. The cover slips were then washed with PBST and incubated with the avidin-peroxidase complex from the Vectastain Universal Elite ABC kit for 20 minutes. Following further PBST wash, the cells were incubated with 3,3'-Diaminobenzidine solution (BioGenex) for 10 minutes and then counterstained with haematoxylin for 3 minutes. After dehydration through 70%, 90% and 100% ethanol, the cover slips were cleared in xylene and mounted on glass slides with mounting medium (DPX, Sigma).

Immunohistochemistry analysis

Immunohistochemistry analysis was performed as previously described [25, 26] using the diagnostic CD30 mouse monoclonal antibody clone H_2 (Dako, M0751) at 1:40 dilution and Vectastain Universal Elite ABC kit (Vector Lab). CD30 cytoplasmic expression was scored as negative (0), weak (1+), medium (2+) and strong (3+) staining.

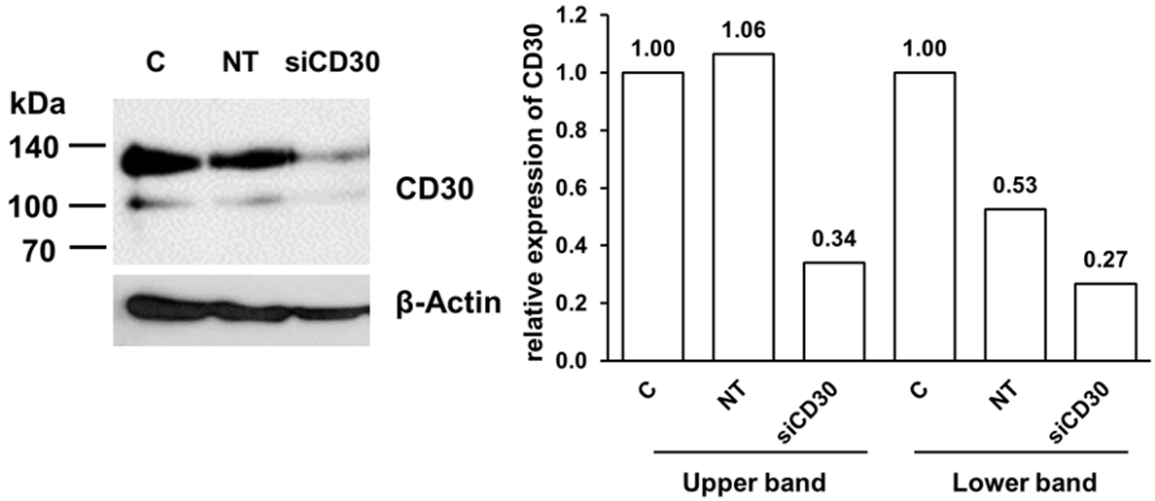


Figure 1. CD30 detection by Western blotting. CD30 antibody specificity test using CD30 knockdown by siRNA in 833K cells (72 hours). Two bands were detected and both correspond to CD30 as they decrease after the knockdown. A representative Western blotting image was shown with quantification of CD30 expression (the high and low molecular weight bands were quantified separately and normalized to the untreated control) presented in bar chart. C: untreated control; NT: Non-targeting control; siCD30: CD30 siRNA treated cells.

Statistics

Dose-response curves for each cytotoxic drug were generated within GraphPad (Non-linear regression, dose-response inhibition, log (inhibitor) vs. response variable slope (4 parameters), ordinary fit). IC30 and IC50 were calculated by using GraphPad QuickCalcs (http://www.graphpad.com/quickcalcs/Ecanything1/) from the dose-response curves. Two tailed Fisher's exact test was applied to category data. $P < 0.05$ was considered as significant.

Results

Determination of CD30 expression in a panel of TGCT cell lines

To analyze CD30 expression levels in both TGCT tissues and cell lines, we first confirmed by Western blot the specificity of the rabbit anti-CD30 antibody using siRNA knockdown of CD30 in 833K cells. Two bands around 100 and 140 KDa were detected and both corresponded to CD30, as they decreased after CD30 knockdown by siRNA (**Figure 1**). These detected bands are consistent with the previously observed sizes of cellular CD30 by Western blotting [27]. We then checked CD30 expression levels in 12 TGCT cell lines, including three cisplatin resistance sublines, by Western blotting and immunocytochemistry. The two detection methods generated consistent CD30 expression results in these cell lines. As expected, the teratoma cell line 577MF was CD30 negative and the seminoma cell line TCam-2 had very low CD30 expression. However, among embryonal carcinoma cell lines, CD30 expression varied dramatically from strong expression in 833K cells to very weak expression in TERA1 cells. The Yolk Sac carcinoma cell line GCT44 was also negative (**Figure 2**). Interestingly, the cisplatin resistant sublines 833KR, SusaR and GCT27R all expressed much lower levels of CD30 than their parental cells and SusaR cells were CD30 negative (**Figure 2B** and **2C**).

Cell killing efficacy of brentuximab vedotin in correlation with CD30 expression

Dose-response curves for brentuximab vedotin were generated in all 12 TGCT cell lines by MTS assay (**Figure 3A** and **3B**). The sensitivity to brentuximab vedotin generally correlated to the CD30 expression in the cell lines. 833K cells, which expressed a high level of CD30, were most sensitive (> 500 folds than others) to brentuximab vedotin. However, NCG2012 cells, which expressed considerable amounts of CD30, as detected both by Western blot and immunocytochemistry analyses, did not respond to brentuximab vedotin until the concentration reached over 5000 ng/ml, which was potentially due to unspecific toxicity at very high drug doses. TCam-2, which expressed CD30

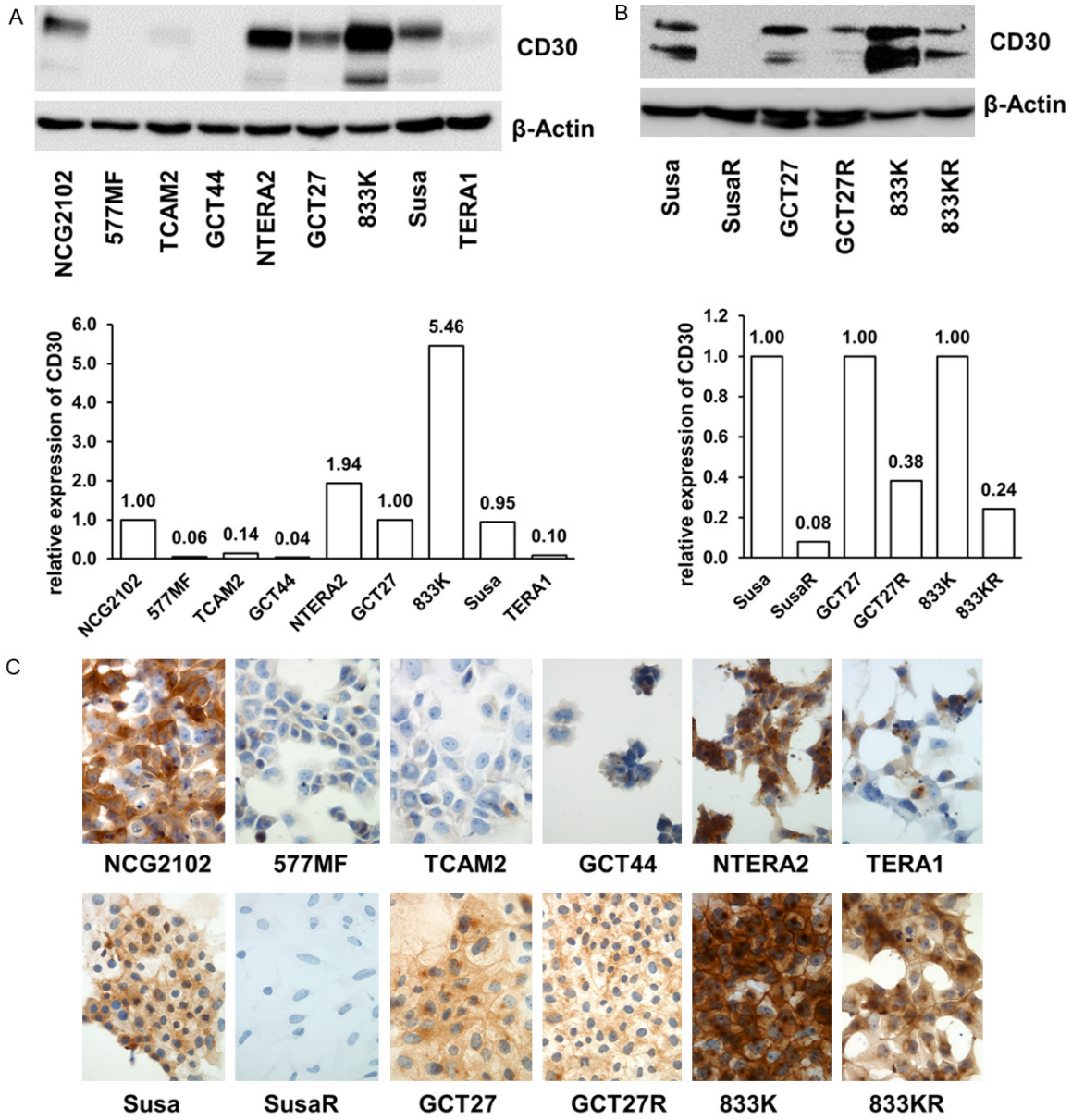
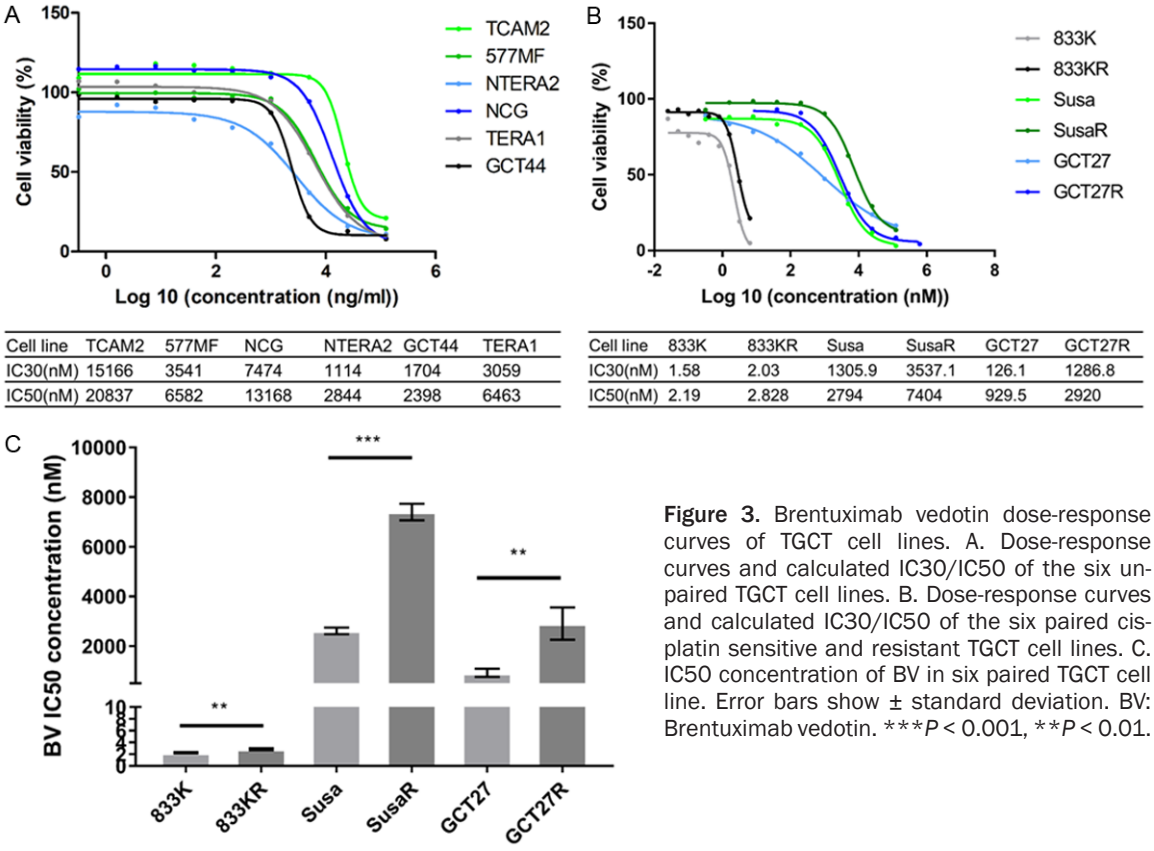


Figure 2. CD30 expression in TGCT cell lines. A. CD30 expression in TGCT cell lines determined by Western blotting with quantification of CD30 expression (both bands normalized to NCG2101) presented in bar chart. B. CD30 expression in the three pairs of cisplatin sensitive parental TGCT cell lines and resistant sublines (SusaR, GCT27R and 833KR) determined by Western blotting with quantification of CD30 expression (both bands normalized to the corresponding cisplatin-sensitive parental cell lines) presented in bar chart. C. Representative images of CD30 expression in these TGCT cell lines determined by immunocytochemistry (400× magnification).

in a small proportion of cells, was another cell line that did not respond to brentuximab vedotin until at > 5000 ng/ml in concentration. The two CD30 negative cell lines, 577MF and GCT44, started to respond to brentuximab vedotin at > 1000 ng/mg, similar to TERA1, which has very low level of CD30 expression. While most cell lines, whether they responded to brentuximab vedotin at low doses or not, are nearly completely killed at 125000 ng/ml concentration in three days, around 20% GCT27 and TCAM2 cells survived at such a high dose of brentuximab vedotin.

The influence of cisplatin treatment on the sensitivity of TGCT cells to brentuximab vedotin

Comparing the 833K, Susa and GCT27 parental cells with the cisplatin resistant sublines, each of the cisplatin resistant cell sublines showed more resistance to brentuximab vedotin



Cell line	TCAM2	577MF	NCG	NTERA2	GCT44	TERA1
IC30(nM)	15166	3541	7474	1114	1704	3059
IC50(nM)	20837	6582	13168	2844	2398	6463

Cell line	833K	833KR	Susa	SusaR	GCT27	GCT27R
IC30(nM)	1.58	2.03	1305.9	3537.1	126.1	1286.8
IC50(nM)	2.19	2.828	2794	7404	929.5	2920

Figure 3. Brentuximab vedotin dose-response curves of TGCT cell lines. A. Dose-response curves and calculated IC30/IC50 of the six unpaired TGCT cell lines. B. Dose-response curves and calculated IC30/IC50 of the six paired cisplatin sensitive and resistant TGCT cell lines. C. IC50 concentration of BV in six paired TGCT cell line. Error bars show ± standard deviation. BV: Brentuximab vedotin. ***$P < 0.001$, **$P < 0.01$.

than its parental line with significantly increased IC50 doses for all the sublines (**Figure 3B** and **3C**). SusaR and GCT27R cells only started to respond to brentuximab vedotin at 1000 ng/ml, while the parental cell lines started to respond at < 10 ng/ml. This brentuximab vedotin sensitivity correlated to the reduced CD30 expression levels in the resistant sublines compared to their parental cells. We further investigated the effects of cisplatin treatment on CD30 expression levels in 833K, Susa and GCT27 cells and observed a significant ($P < 0.01$ for all cell lines) reduction in CD30 expression after treatment as detected by Western blotting (**Figure 4A**) and immunocytochemistry analysis (**Figure 4B**). We then tested the combined treatment of brentuximab vedotin and cisplatin for 833K, Susa and GCT27 cells using both IC30 and IC50 of cisplatin and brentuximab vedotin in different dose combinations. An antagonistic effect was detected in all three cell lines (**Figure 4C**).

CD30 expression stimulation in TGCT cells

As CD30 expression is low or negative in some cell lines and post-chemotherapy TGCT embryonal carcinoma clinical samples, we attempted to induce CD30 in TGCT cells by different methods. Firstly, we treated GCT27 and TERA1 cells with the DNA demethylation agents Cladribine and 5-Aza-2'-deoxycytidine. To our surprise, both agents decreased instead of increasing CD30 expression, as determined by Western blot (**Figure 5A**). We then tested the effect of CD30 ligand and methotrexate treatments for their potential in stimulating CD30 expression in TGCT cells using 833K and GCT27 cell lines. Both drug treatment increased CD30 expression in 833K cells, but reduced CD30 expression in GCT27 cells (**Figure 5B**).

Determination of the efficacy of combination treatment of brentuximab vedotin with chemotherapies

Based on the above data, the combination treatment of brentuximab vedotin with other chemotherapy drugs used in the clinic for TGCT treatment, including methotrexate, etoposide, paclitaxel, SN-38 and actinomycin-D, were tested in 833K, Susa and GCT27 cell lines to determine which drugs have a similar antagonistic effect to brentuximab vedotin as cisplatin and,

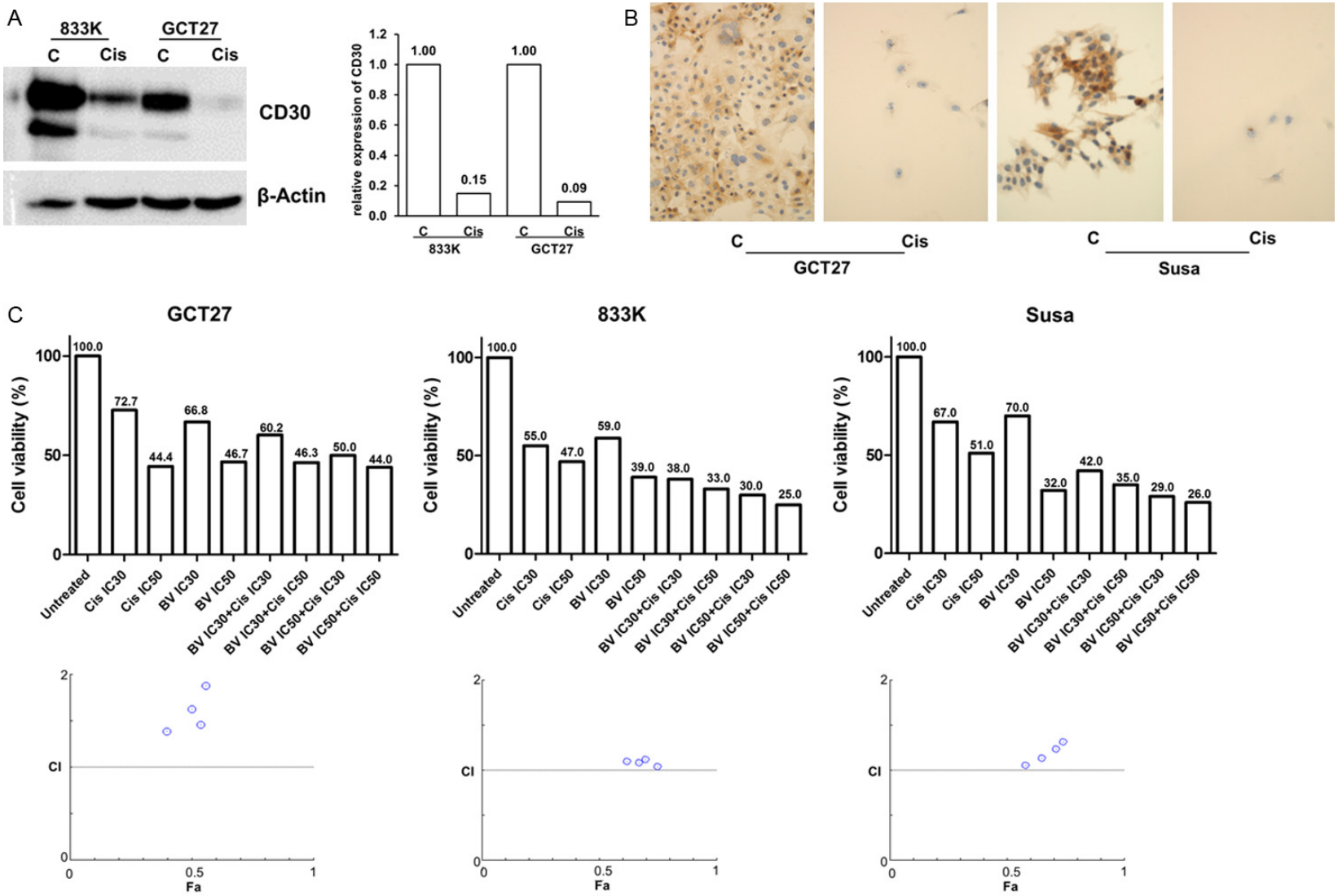


Figure 4. The effect of cisplatin on CD30 expression and brentuximab vedotin sensitivity of TGCT cells. A. Representative Western blotting images and the quantifications of CD30 normalized to the untreated control cells show the reduction of CD30 expression in TGCT cells after cisplatin treatment. B. Representative immunocytochemistry images of the reduction of CD30 expression in TGCT cells after cisplatin treatment. C. Antagonism of cisplatin and Brentuximab vedotin in the treatment of CD30 positive TGCT cells with cell viability bar chart on the top panel and combination index plot in the bottom panel. C: untreated control; Cis: cisplatin treated cells; BV: Brentuximab vedotin; CI: combination index; Fa: Fraction affected.

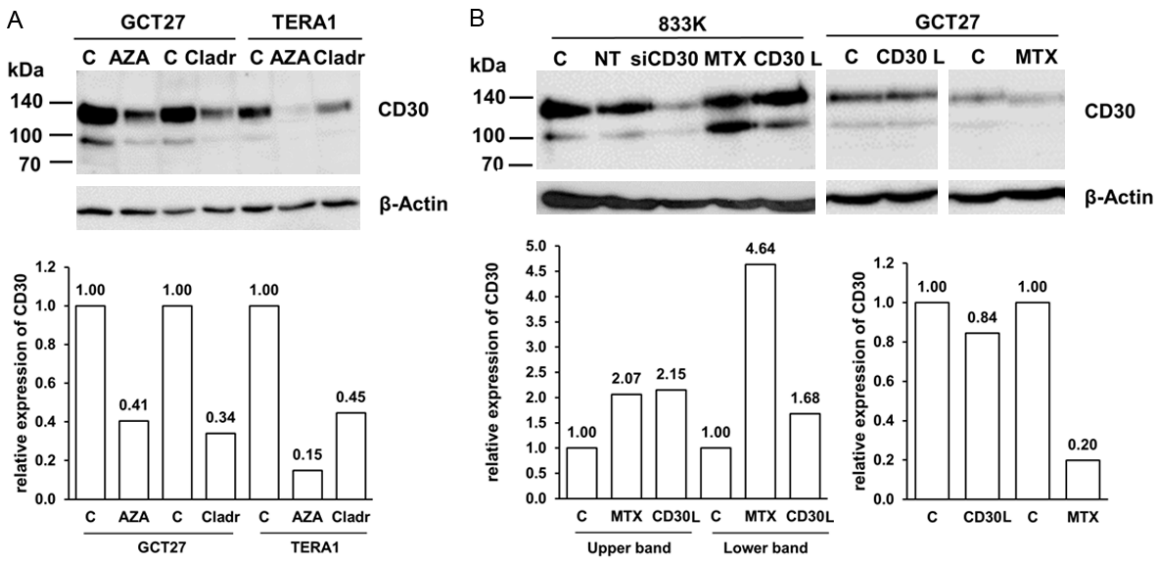


Figure 5. The impact of previously reported CD30 reactivation agents on CD30 expression in TGCT cells. A. Western blotting images of CD30 expression in TGCT cells treated using DNA demethylation agents 5-Aza-2'-deoxycytidine and cladribine with the quantification of CD30 expression (both bands normalized to untreated control cells) presented in bar chart. B. Western blotting images of CD30 expression in TGCT cells treated using methotrexate and CD30 Ligand. In 833K cells, the Western blotting was run together with the CD30 knockdown experiment by siRNA as presented in **Figure 1**. The high and low molecular weight bands of CD30 were quantified separately in 833K and normalized to untreated control cells. In GCT27, the quantification of both bands of CD30 was normalized to the control cells. C: untreated control. AZA: 5-Aza-2'-deoxycytidine treated cells; Cladr: cladribine treated cells; MTX: methotrexate treated cells; CD30 L: CD30 Ligand treated cells.

more importantly, which drugs have synergistic or additive effects to brentuximab vedotin. In order to select the dose for the combined treatment, we firstly generated the dose-response curves for each of these drugs in 833K, Susa and GCT27 cell lines (**Figure 6**). Interestingly, we observed a unique dose-response effect for methotrexate, different from all the other drugs. Methotrexate did not affect the proliferation of cancer cells until reaching certain concentrations, after which a small increase in methotrexate dose dramatically inhibited cancer cell growth and further increases in the dose had no additional effect (**Figure 6**). Consequently, all three cell lines were not affected by 10 nM methotrexate but were maximally inhibited at a 100 nM dose. To determine if this dose response pattern of methotrexate is universal for any cancer type, we investigated four prostate cancer cell lines PC3, LNCaP, 22RV1 and DU145 and an osteosarcoma cell line MG63 for their methotrexate dose responses. All these cell lines also showed plateauing of methotrexate inhibiting effects from certain drug concentrations, ranging from 50-500 nM in different cell lines. More than 20% of cells survived drug concentration > 100-fold of the minimum plateauing dose. However, the minimum plateauing doses for these cells were reached gradually with increasing methotrexate doses in a range of 10-fold difference (**Figure 7**).

Once IC30 and IC50 were calculated for all drugs, combination treatments with brentuximab vedotin were performed using both IC30 and IC50 for different dose combinations. Cell viability (%), killing effect (from 0 to 1) and synergy for methotrexate, etoposide, paclitaxel, SN-38 and actinomycin-D are shown in **Figures 8-10**. Based on the synergy analysis using CompuSyn software, brentuximab vedotin showed antagonism with all the tested cytotoxic drugs in GCT27 cells and with methotrexate in all cell lines. Weak antagonistic effects were also detected for etoposide in 833K cells and for actinomycin-D in Susa cells. Weak synergy with paclitaxel in 833K and Susa cells and with etoposide in Susa cells were observed. Additive effects were observed for SN-38 in 833K and Susa cells and actinomycin-D in 833K cells (**Figures 8-10**).

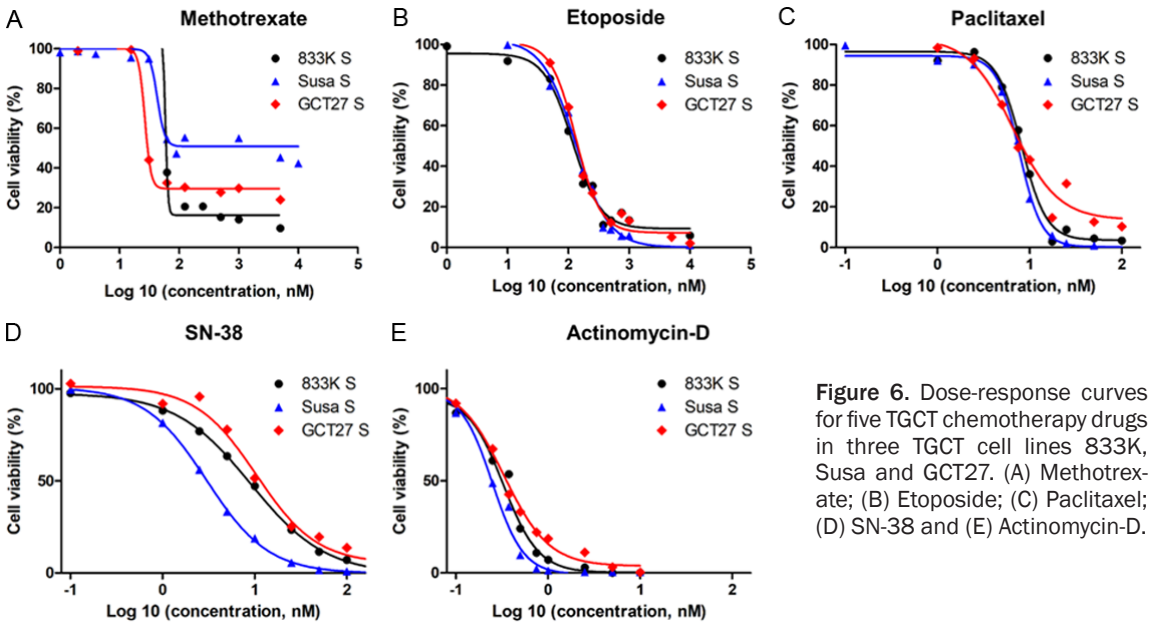
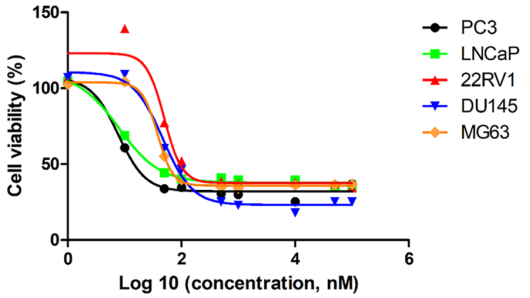


Figure 6. Dose-response curves for five TGCT chemotherapy drugs in three TGCT cell lines 833K, Susa and GCT27. (A) Methotrexate; (B) Etoposide; (C) Paclitaxel; (D) SN-38 and (E) Actinomycin-D.



Cell line	PC3	LNCaP	22RV1	DU145	MG63
IC30(nM)	4.83	3.92	36.69	28.13	29.03
IC50(nM)	7.715	7.9	49.71	46.76	37.77

Figure 7. MTX dose-response in prostate cancer and the MG63 osteosarcoma cell lines and the calculated IC30/IC50.

Determination of the CD30 expression in post-radiotherapy TGCT lesions

We performed immunohistochemistry analysis to detect CD30 expression in a large cohort of post-radiotherapy clinical TGCT samples made into TMAs, including 57 non-seminomous TGCTs with pure or areas of embryonal carcinoma and 34 mixed seminoma and seminomous TGCTs with embryonal carcinoma components, one pure teratoma, three cases of choriocarcinoma mixed with seminoma and three cases of teratoma mixed with seminoma. In 91% (52/57) of non-seminomous TGCTs with embryonal carcinoma components, CD30 expression was found in > 10% of tumor cells. In 94% (32/34) of mixed seminoma and embryonal carcinoma/teratoma TGCT cases, CD30 expression was found in > 10% of tumor cells. The former type of TGCTs had the trend to express CD30 stronger than the mixed tumors (more 2+/3+ cases, $P = 0.096$) (**Table 1**; **Figure 11**). The pure teratoma was CD30 positive, two of the three choriocarcinomas were CD30 positive and the three teratomas mixed with seminoma cases were all CD30 negative.

Discussion

Brentuximab vedotin has been approved by FDA for the treatment of CD30 positive Hodgkin lymphoma and anaplastic large cell lymphoma [5] with a good tolerability and safety profile. Most TGCTs express CD30, in particular embryonal carcinomas [9-15]. Therefore, CD30 positive TGCTs could benefit from brentuximab vedotin therapy. Two clinical studies have demonstrated in limited number of patients the potential benefit of this treatment in post-chemotherapy relapsed TGCTs. However, only a small proportion of patients responded [17, 18]. Further investigations are required to improve the efficacy of brentuximab vedotin in TGCT treatment. Therefore, we determined the prevalence of CD30 expression in a considerable large panel of TGCT cell lines to establish the sensitivity of TGCT cells to brentuximab vedotin, in correlation to CD30 expression levels. We also explored the combined treatment of brentux-

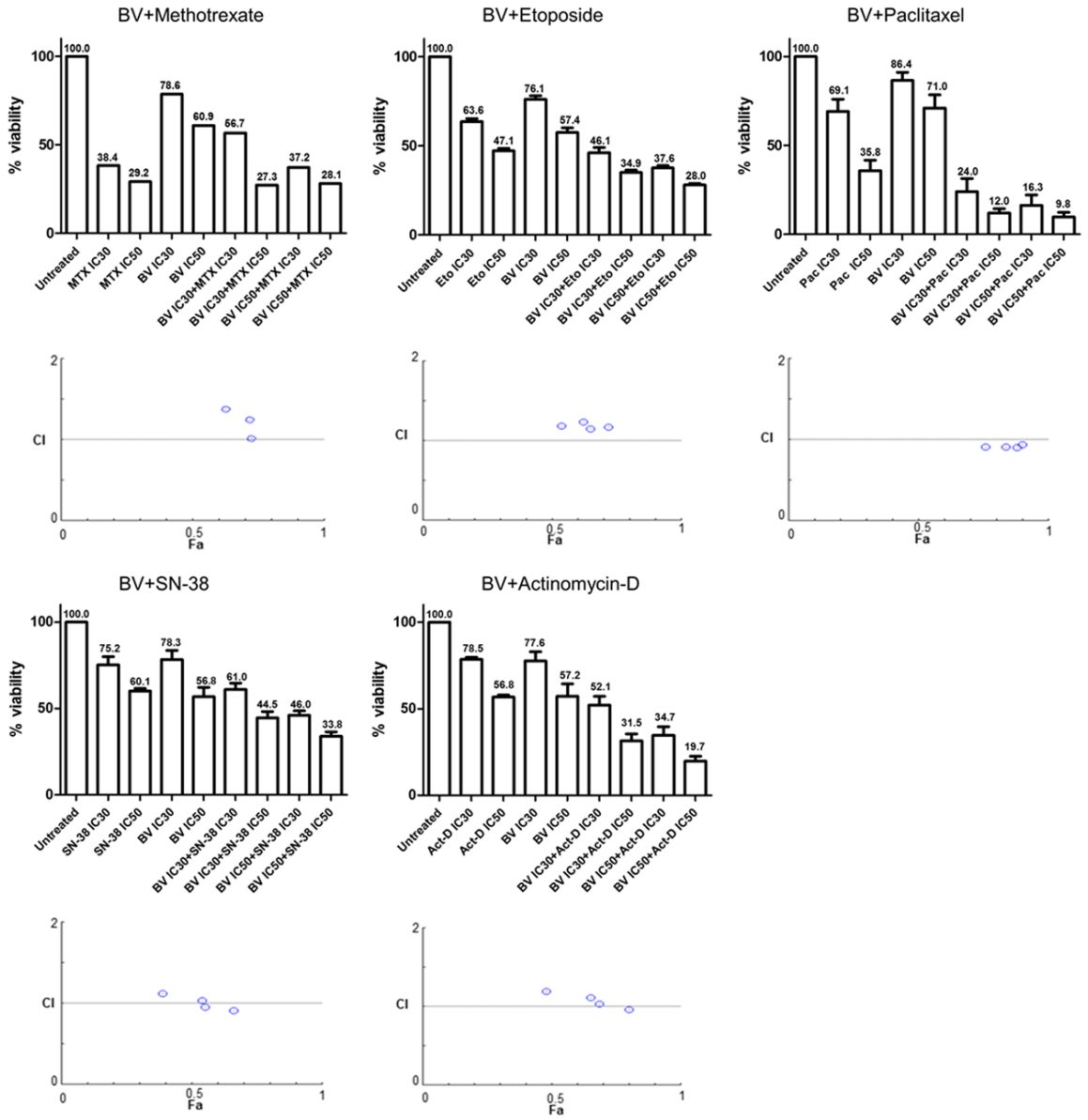


Figure 8. The efficacy of brentuximab vedotin in combination with other TGCT chemotherapy drugs in 833K cells. Brentuximab vedotin was combined with methotrexate, etoposide, paclitaxel, SN-38 and actinomycin-D for the treatment of 833K cells with the cell viability bar chart showing on the top of the combination index plot for each treatment. The antagonistic effect of brentuximab vedotin IC30 and methotrexate IC30 combination is out of range, so that there are only three data points shown in the combination index plot for brentuximab vedotin and methotrexate. MTX: methotrexate; BV: brentuximab vedotin; Eto: etoposide; Pac: paclitaxel; ActD: actinomycin-D; CI: combination index; Fa: Fraction affected.

imab vedotin with currently used clinical chemotherapy drugs for TGCT treatment to establish the best combination to maximally kill CD30 positive TGCTs.

We have shown that in the only existing seminoma cell line TCAM2, CD30 is only expressed in a small proportion of cells, and is not expressed in the Yolk Sac carcinoma cell line GCT44, which is consistent with previous studies [8, 28]. In a panel of embryonal carcinoma TGCT cell lines, we detected a wide range of CD30 expression levels, which provides good *in vitro* models to study the sensitivity to brentuximab vedotin with respect to CD30 levels and to investigate potential mechanisms of

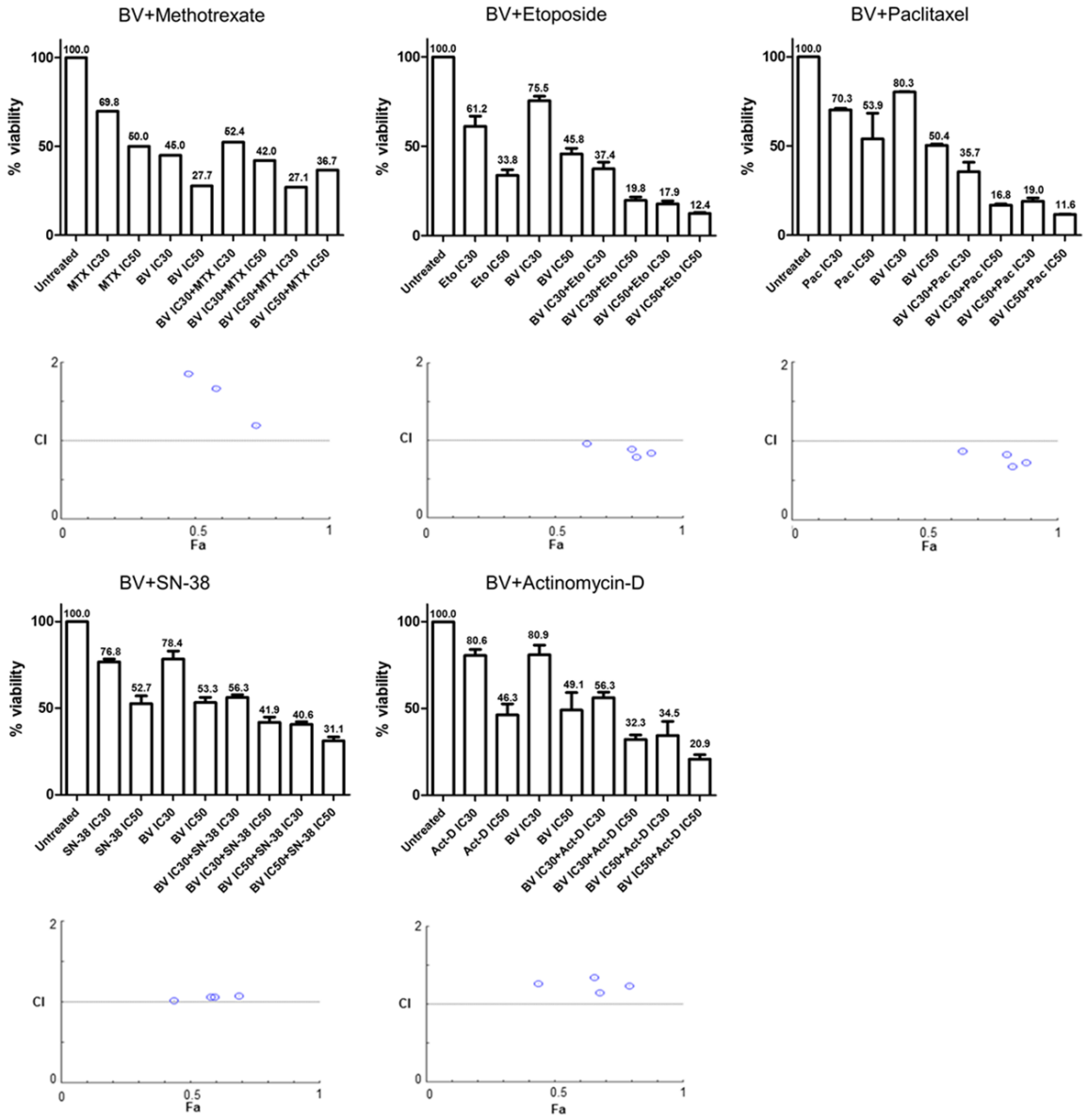


Figure 9. The efficacy of Brentuximab vedotin in combination with other TGCT chemotherapy drugs in Susa cells. Brentuximab vedotin was combined with methotrexate, etoposide, paclitaxel, SN-38 and actinomycin-D for the treatment of Susa cells with the cell viability bar chart showing on the top of the combination index plot for each treatment. The antagonistic effect of brentuximab vedotin IC50 and methotrexate IC50 combination is out of range, so that there are only three data points shown in the combination index plot for brentuximab vedotin and methotrexate. MTX: methotrexate; BV: brentuximab vedotin; Eto: etoposide; Pac: paclitaxel; ActD: actinomycin-D; CI: combination index; Fa: Fraction affected.

resistance. While we observed a general correlation of TGCT cell sensitivity to brentuximab vedotin and their CD30 expression levels, exceptions exist. The cell line with the second highest CD30 expression was amongst the least sensitive cell lines to brentuximab vedotin, indicating that the sensitivity to brentuximab vedotin is also dependent on other factors. It has been reported that in a Hodgkin lymphoma cell line, the induced brentuximab vedotin resistance is caused by increased expression of Multidrug resistance protein 1 (MDR1) which facilitates the export of MMAE out of the cells [29]. MDR1 expression has been associated with advanced stage TGCTs [30], therefore, its expression should be con-

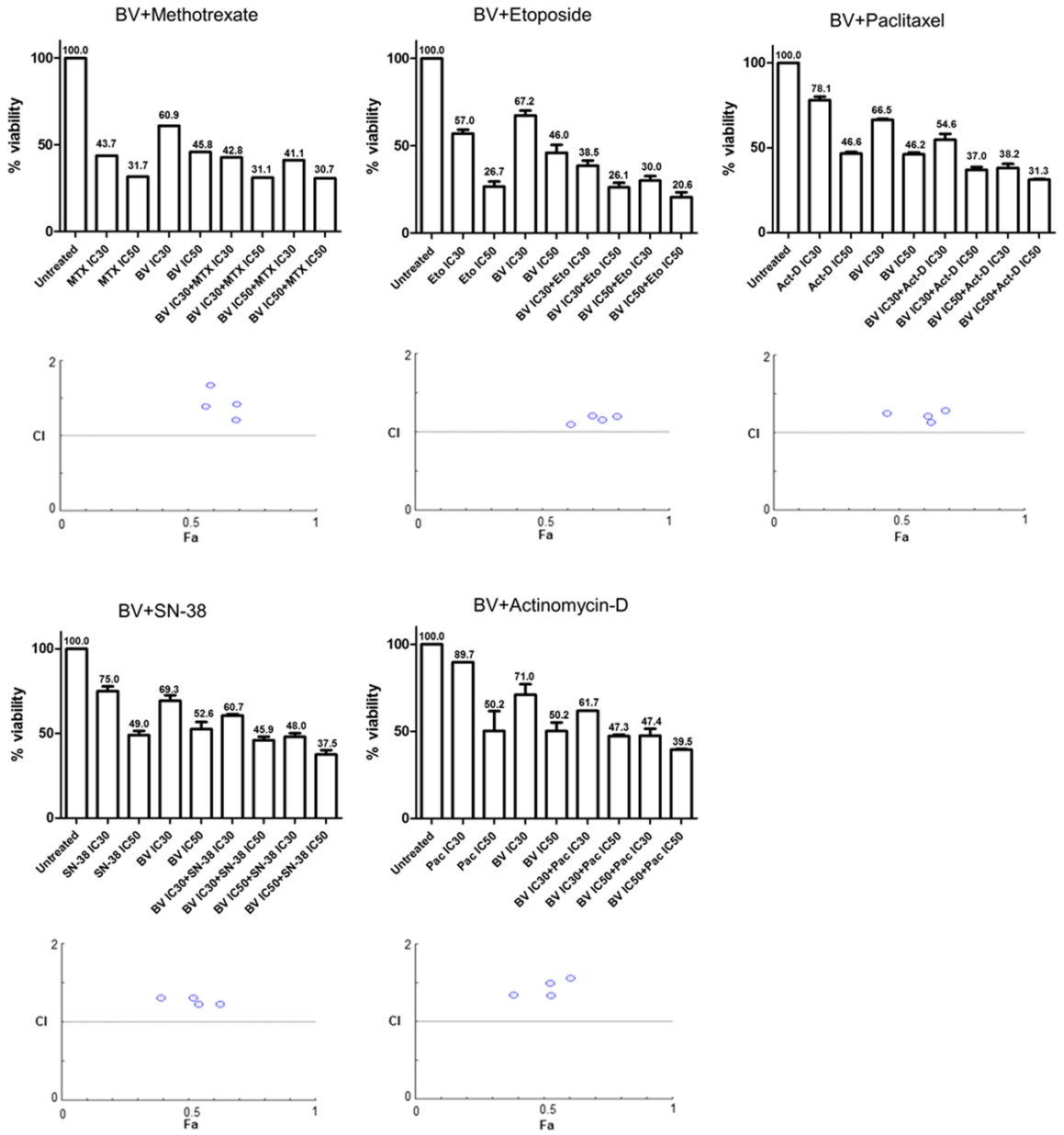


Figure 10. The efficacy of Brentuximab vedotin in combination with other TGCT chemotherapy drugs in GCT27 cells. Brentuximab vedotin was combined with methotrexate, etoposide, paclitaxel, SN-38 and actinomycin-D for the treatment of GCT27 cells with the cell viability bar chart showing on the top of the combination index plot for each treatment. MTX: methotrexate; BV: brentuximab vedotin; Eto: etoposide; Pac: paclitaxel; ActD: actinomycin-D; CI: combination index; Fa: Fraction affected.

sidered in the clinical application of brentuximab vedotin for TGCT therapy. Other chemotherapy drug resistance mechanisms may also contribute to brentuximab vedotin resistance, which remain to be determined.

Importantly, we have three pairs of parental and cisplatin resistant non-seminoma sublines [20, 31-33], that allowed the study of brentuximab vedotin response in association with the treatment and sensitivity to cisplatin, the primary cytotoxic chemotherapeutic drug for TCGT patient treatment. We found that all the cisplatin resistant sublines were more resistant to brentuximab vedotin treatment and cisplatin treatment of CD30 positive TGCT cell lines selectively killed CD30 positive cells. These data indicate that the previously observed reduction

Table 1. CD30 expression in post-radiotherapy TGCT samples determined by immunohistochemistry staining

Stain level	0	1+	2+	3+
Mixed tumor with EC component	2	12	15	5
NS with EC component	5	8	27	17

EC: embryonal carcinoma; NS: non-seminomous testicular germ cell tumor.

in CD30 positive rate in post-chemotherapy clinical TGCT samples [11, 15] may be largely due to platinum drugs, which are commonly included in TGCT treatment. As brentuximab vedotin kills not only CD30-expressing cancer cells, but also surrounding CD30 negative cancer cells through a bystander effect [6-8], this raises the question of whether brentuximab vedotin should be used before or after platinum-based chemotherapy in clinical trials. Brentuximab vedotin had been applied after chemotherapy in the only two reported small-scale clinical studies, with clear but limited benefit. Based on our *in vitro* data and the known low toxicity of brentuximab vedotin, we would propose the application of brentuximab vedotin before platinum-based chemotherapy in future clinical trials of CD30+ advanced stage TGCTs. A pilot phase II trial with brentuximab vedotin treatment before any other therapies in Hodgkin lymphoma showed encouraging results [34].

While chemotherapy selectively kills CD30 positive TGCT cells, leading to reduced CD30 expression in the resistant tumor cells and brentuximab vedotin resistance, certain agents such as DNA methylation inhibitors, CD30 ligand and methotrexate have been reported to induce CD30 expression [35-38]. Therefore, a potential therapeutic option would be to induce CD30 expression in negative or low-expressing tumors. It has been reported by Hasanali *et al.* that CD30 expression can be reactivated in T-cell prolymphocytic leukemia by demethylating agent Cladribine and, consequently, induce tumor cell sensitivity to brentuximab vedotin [35]. However, in contrast with the previous observation in leukemia, we found that both DNA demethylation agents, Cladribine and AZA-C decreased, instead of increasing, CD30 expression. In the study by Hasanali *et al.*, it was shown that Cladribine induced CD30 expression in T-cell prolymphocytic leukemias, but not in leukemic mantle cell lymphomas [35]. CD30 ligand has been shown to upregulate CD30 through a positive feedback loop through NFKB and IRF4 [36]. Our analysis in two TGCT cell lines showed slightly differences in CD30 expression before and after the treatment but in opposite directions. Further investigations are required.

Methotrexate has been reported to activate CD30 in certain lymphomas [37, 38]. In our study of two TGCT cell lines, CD30 expression was increased in one cell line and decreased in the other after methotrexate treatment, thus, it is hard to make conclusion. Our combined treatment with brentuximab vedotin and methotrexate showed antagonistic effects in both of these cell lines as well as another cell line, suggesting that simultaneous treatment with these two drugs of TGCT patient should be avoided. However, the potential to stimulate CD30 expression using methotrexate before brentuximab vedotin should be further investigated. Interestingly, we found that methotrexate has a unique dose-response effect in TGCT cells. At a certain dose point, the maximum tumor cell killing effect can be achieved by only a slight dose increase from the dose where almost no effect occurs. Generally, cancer cells within a case or cell line are heterogeneous in their sensitivity to a cytotoxic drug and the amount of cells killed increases in proportion to drug doses. Our TGCT methotrexate dose-response curves suggest that there are only two distinct populations in each TGCT cell line we tested. One population of cells can be killed at certain doses of methotrexate and the other population of cell can never be killed by methotrexate no matter how high the concentration is. This not only suggests that TGCT cells provide a good model to study methotrexate drug resistance mechanisms, but also indicates the potential to find a minimum dose for individual patients that would minimalize methotrexate side effects without sacrificing the maximum treatment benefit.

Most importantly, we investigated the efficacy of combinations of brentuximab vedotin with a large number of the chemotherapy drugs commonly used in the clinic for TGCT treatment. Currently, there is limited data on treatment efficacy of combined brentuximab vedotin and chemotherapeutic drugs commonly used for TGCT patient treatments. While certain com-

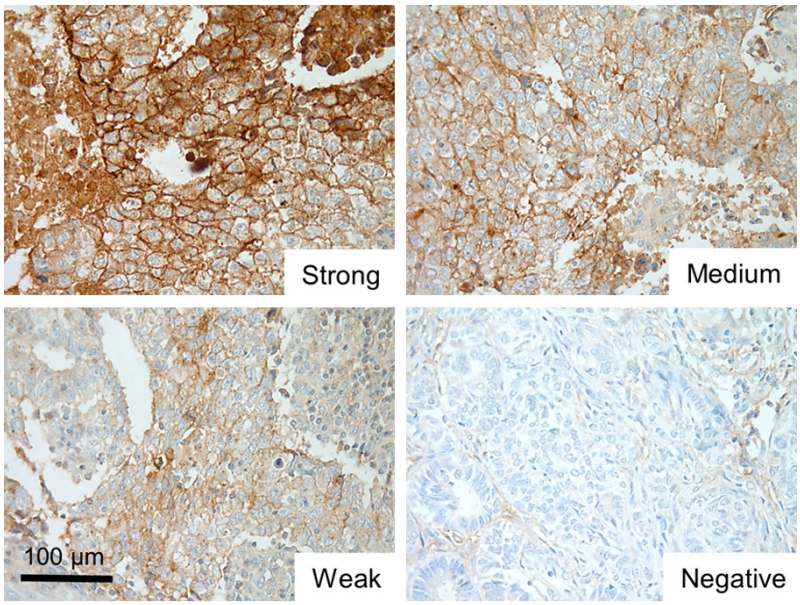


Figure 11. CD30 immunochemistry staining in TGCT clinical samples. Representative images from CD30 negative, weak, medium and strong positive TGCT cases were shown.

monly used chemotherapy drugs showed antagonism, we found synergistic/additive effects for some drugs, such as etoposide, paclitaxel and SN-38. Therefore, combining brentuximab vedotin with these non-platinum-based chemotherapies may be an effective therapeutic approach for patients with advanced stage CD30 positive TGCTs. For example, brentuximab vedotin may be used together with paclitaxel and after 48 hours a platinum drug can be added to avoid any direct antagonism.

Surprisingly, we found that all chemotherapy drugs in combination with brentuximab vedotin showed antagonism in GCT27 cells while half of them showed synergistic/additive effects in 833K and Susa cells, indicating that GCT27 cells have certain unique features affecting their response to brentuximab vedotin. GCT27 cells are commonly used for TGCT *in vitro* studies, including previous studies of brentuximab vedotin [8], due to their easy to culture nature. Our data suggest that cautions should be taken to generalize findings from GCT27 cells for brentuximab vedotin studies.

As brentuximab vedotin has been used for the treatment of lymphomas with a good safety profile, for those drugs where synergy and or additive effects were observed, the combined treatment will be ready to translate into a clinical trial to investigate the clinical efficacy in treating advanced TGCTs. Patients with any advanced CD30 positive TGCTs would be eligible to receive brentuximab vedotin at the current approved dose. In this group of patients treatment response can be monitored with already established tumor markers. In addition, as detected by PET scanning, they are highly glucose avid and early responses can be seen within 21 days of the start of therapy. Therefore, an initial 3 weeks of brentuximab vedotin treatment can be followed by a PET scan. If the clinical response was defined as being suboptimal on PET scan, the combined treatment with one of the synergistic/additive effect chemotherapy drugs revealed from this study may be applied.

Two separate case reports of combination treatment by brentuximab vedotin and radiotherapy of advanced chemotherapy resistant Hodgkin lymphoma showed good efficacy of the combined treatment [39, 40]. In one case, complete remission at six months was achieved when the case was reported [40]. We determined the prevalence of CD30 expression in a large cohort of post-radiotherapy non-seminomous TGCT clinical samples, including mixed seminoma and non-seminomous tumors. In > 90% of cases, CD30 expression remained in post-radiotherapy tumor tissues. This indicates that brentuximab vedotin will be effective after or in combination with radiotherapy. Although this combination treatment may not benefit many TGCT patients, since radiotherapy is currently only used in patients with multiple relapses, it might be useful in other CD30 positive malignant diseases where radiotherapy is commonly used.

In summary, we evaluated the therapeutic efficacy of brentuximab vedotin in correlation with CD30 expression in TGCT cell lines. We revealed that cisplatin chemotherapy selectively kills

CD30 positive TGCT cells, making it a poor choice to treat TGCTs after or in combination with cisplatin. We also identified combinations of brentuximab vedotin with certain chemotherapy drugs with synergetic or additive effects. All these data support the clinical evaluation of brentuximab vedotin to treat TGCTs and shed light on the optimal combinational therapeutic strategies.

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Disclosure of conflict of interest

None.

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