**In vitro** sensitivity testing of minimally passaged and uncultured gliomas with TRAIL and/or chemotherapy drugs

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TRAIL/Apo-2L has shown promise as an anti-glioma drug, based on investigations of TRAIL sensitivity in established glioma cell lines, but it is not known how accurately TRAIL signalling pathways of glioma cells in vivo are reproduced in these cell lines in vitro. To replicate as closely as possible the in vivo behaviour of malignant glioma cells, 17 early passage glioma cell lines and 5 freshly resected gliomas were exposed to TRAIL-based agents and/or chemotherapeutic drugs. Normal human hepatocytes and astrocytes and established glioma cell lines were also tested. Cross-linked TRAIL, but not soluble TRAIL, killed both normal cell types and cells from three tumours. Cells from only one glioma were killed by soluble TRAIL, although only inefficiently. High concentrations of cisplatin were lethal to glioma cells, hepatocytes and astrocytes. Isolated combinations of TRAIL and chemotherapy drugs were more toxic to particular gliomas than normal cells, but no combination was generally selective for glioma cells. This study highlights the widespread resistance of glioma cells to TRAIL-based agents, but suggests that a minority of high-grade glioma patients may benefit from particular combinations of TRAIL and chemotherapy drugs. In vitro sensitivity assays may help identify effective drug combinations for individual glioma patients.

**Keywords:** glioma; astrocytoma; glioblastoma; Apo-2L; apoptosis

The vast majority of malignant glioma patients die within 2 years of diagnosis, regardless of treatment (Group, 2002; Stupp et al., 2005). More effective treatments are therefore urgently required. Chemotherapy and irradiation trigger apoptosis of sensitive cells by provoking the ‘intrinsic’ apoptosis pathway (Norbury and Johnston, 2005). This involves the detection of DNA damage and instigation of a self-destruction program, which is regulated by the Bcl-2 family and executed by a molecular machinery including cytochrome c, Apaf-1 and the apoptotic proteases caspase-9 and caspase-3. Defects in the intrinsic pathway can contribute to resistance to chemotherapy and radiotherapy (Longley and Johnston, 2005). In contrast, ‘death ligands’, members of the TNF-α superfamily including FasL/CD95 and TRAIL/Apo2L, stimulate apoptosis through the ‘extrinsic’ pathway (Thorburn, 2004). Ligation of death receptors (such as Fas, DR4/TRAIL-R1 and DR5/TRAIL-R2) promotes recruitment of an adaptor molecule, FADD, caspase-8 and/or caspase-10 to form a complex known as the death-inducing signalling complex (DISC) (Kischkel et al., 1995). The initiator caspases are activated within the DISC, and acquire the ability to proteolytically activate effector caspases (such as caspase-3), either directly or indirectly (Scaffidi et al., 1998). The downstream caspases then destroy the cell by digesting numerous cellular proteins.

Because this extrinsic apoptosis pathway uses distinct components from that triggered by conventional anti-cancer treatments, there has been substantial research interest in exploiting its potential for treating tumour types that are unresponsive to currently available therapies. TRAIL and agonistic anti-TRAIL receptor antibodies are currently being evaluated in early-phase clinical trials. Initial reports portrayed TRAIL as an exemplary anti-cancer agent, as it induced apoptosis in many types of tumour cells but, unlike FasL, did not kill normal cells. Subsequent studies tempered that initial optimism somewhat. Although the extra-cellular portion of human TRAIL (amino acids 114–281, henceforth referred to as soluble TRAIL) was generally tolerated by normal human cells (Ashkenazi et al., 1999), other formulations were found to be toxic to particular normal cell types (Leverkus et al., 2000; Nitsch et al., 2000; Pettersen et al., 2002). Freshly isolated human hepatocytes displayed substantial sensitivity to His-tagged TRAIL, cross-linked TRAIL formulations and agonistic
anti-DR4 and DR5 antibodies (Jo et al., 2000; Mori et al., 2004; Ganten et al., 2006), but survived incubation with soluble TRAIL (Askenazi et al., 1999; Lawrence et al., 2001; Ganten et al., 2006). The TRAIL sensitivity of normal brain cells is particularly relevant for the development of TRAIL-based anti-glioma therapies. Human astrocytes were relatively resistant to soluble untagged TRAIL in vitro (Askenazi et al., 1999; Song et al., 2006). Cell death was detected in brain slices incubated with FLAG-tagged TRAIL that had been cross-linked with an anti-FLAG antibody (Nesterov et al., 2002). Immunofluorescent assays suggested that the cells killed in these experiments included isolated neurons, oligodendrocytes, astrocytes and microglial cells (Nesterov et al., 2002).

Established glioma cell lines vary considerably in their responsiveness to TRAIL receptor ligation (reviewed in Hawkins, 2004). It is presently unknown how faithfully the death ligand signalling pathways of glioma cells in vivo are mimicked by established glioma cell lines, but it has been shown that glioma cells do undergo substantial phenotypic changes in vitro (Anderson et al., 2002; Lee et al., 2006). TRAIL sensitivity of freshly resected uncultured glioma cells has not been reported to date. A few papers have documented the TRAIL responsiveness of minimally cultured gliomas, most of which were resistant to TRAIL as a sole agent (Roa et al., 2003; Song et al., 2003; Jeremias et al., 2004; Eramo et al., 2002b), Li et al., (2006; Koschny et al., 2007). TRAIL can cooperate with other agents, including currently used chemotherapy drugs, to kill established glioma cells that survive exposure to TRAIL alone. It was recently published that the proteosomal degradation of Bcl-xL and TRAIL receptor levels in resistant glioma cell lines (Rieger et al., 2001; Ashley et al., 2002) was mimicked by the combination of TRAIL/chemotherapy treatment for malignant glioma, this study examined the responses of glioma cells, astrocytes and hepatocytes to TRAIL-based agents and/or chemotherapy drugs. Four TRAIL-related agents were tested: two forms of cross-linked TRAIL (F-LZ-TRAIL and ‘Superkiller’), the extracellular portion of TRAIL (‘soluble TRAIL’) and an agonistic anti-DR5 antibody. Seven chemotherapy drugs used for glioma therapy were also employed: cisplatin, carboplatin, CCNU, temozolomide, etoposide, vincristine and procarbazine. The drug combinations were tested on freshly resected gliomas and early passage glioma cell lines, to mimic as closely as possible the in vivo behaviour of malignant glioma cells.

MATERIALS AND METHODS

Glioma samples and normal cells

Table 1 provides details about the patients whose tumours were assayed in this study. Gliomas RMH018-023 were resected at the Royal Melbourne Hospital, Australia. Informed consent was obtained from the patients, and approval for this study was obtained from the ethics committees of the Royal Children’s Hospital, Royal Melbourne Hospital and La Trobe University. To generate a single cell suspension, tumour pieces were minced with a scalpel, then incubated with Accumax (Sigma, St Louis, MO, USA) and filtered through a tea stainer and 100 μm filter. Viable cells were isolated by Ficoll density centrifugation.

The ‘D’ series of early passage lines was derived from specimens obtained from patients who had undergone tumour resection at Duke University Hospital (Durham, NC, USA). Informed consent was obtained from each patient prior to surgery in accordance with Duke Internal Review Board stipulations. The tumour material was collected in DMEM, 10% foetal bovine serum (FBS), 0.05 mg ml⁻¹ gentamycin. Tumour samples were drained, placed in a 100 mm tissue culture dish and minced with sterile scissors. Warm sterile-filtered 0.4% collagenase solution (0.4% collagenase, 0.05 mg ml⁻¹ gentamycin in zinc option-MEM, ZO-MEM) (Invitrogen, Carlsbad, CA, USA) was added to the minced tissue and incubated at 37°C for 1 h. Collagenase solution was inactivated by the addition of ZO-MEM, 10% FBS, 0.05 mg ml⁻¹ gentamycin. Minced tissue was titrated to further homogenise sample and then centrifuged (1000 r.p.m., 5 min). Collagenase and media were removed, cells were re-suspended in fresh media and transferred to a 60-mm tissue culture dish and incubated at 37°C, 5% CO₂. Cell cultures with a large RBC fraction were treated with haemolysis solution (0.83% ammonium chloride) as follows: cells were trypsinized and centrifuged (1000 r.p.m., 7 min), supernatant was discarded and cells were re-suspended in 2 ml FBC. Haemolysis solution was added at a ratio of 1:5 to 1:10. Haemolysis mixture was incubated at 4°C for 10 min, additional FBS was then added to bathe cells. Solution was centrifuged (1000 r.p.m., 7 min), following which the supernatant was discarded and the remaining cells were re-suspended in ZO-MEM, supplemented as described earlier. Tumour cultures were serially passaged using 0.25% trypsin-EDTA and collected in freezing medium (12.5% DMSO, 50% FBS and 37.5% ZO-MEM).

The early passage lines LM-G-2, LM-G-4 and LM-G-8 were made from tumours resected at the Austin Hospital (Heidelberg, VIC, Australia). Patients with known or suspected glioblastoma multiforme (GBM) were prospectively entered into a clinical trial after written informed consent was obtained. Ethics approval was granted by the Austin Hospital Human Research Ethics Committee. After intra-operative confirmation of a diagnosis of GBM, fresh tissue samples were obtained by biopsy or resection of tumour. Samples were mechanically disaggregated using the Medimachine (DAKO Diagnostika GmbH, Hamburg, Germany) (Brockhoff et al., 1999) and introduced into pre-warmed DMEM (Life Technologies, Grand Island, NY, USA) containing 10% FBS (CSL, Melbourne, VIC, Australia), 2 μg ml⁻¹ glutamine (Sigma Chemical Co, St Louis, MO, USA) and 0.05 μg ml⁻¹ penicillin/50 μg ml⁻¹ streptomycin, respectively (Life Technologies). After 24 h, any non-adherent cells and material were discarded and the media replenished. Media were replenished twice weekly or more frequently if required. When cells appeared to have reached 50% confluence or maximal confluency in a T25 flask for that cell line, they were expanded into a T75 flask (passage 2). Cells were also expanded into a T75 (passage 3) flask when they reached 50%
### Table 1  Patient and cell line features

| Tumour/Cell line | Sex, age (years) | Tumour grade (WHO) | Passage number | p53 genotype | Treatment before sample obtained | Treatment after sample obtained | Progression free survival | Patient status |
|------------------|------------------|--------------------|----------------|--------------|----------------------------------|-------------------------------|--------------------------|----------------|
| D2234            | M, 52            | IV                 | 10             | M            | Radiotherapy, temozolomide       | BCNU wafer, O6BG, cloretazine, AP23573 | 2 months                 | Died 7 months post-resection |
| D2235            | F, 20            | III                | 9              | M            | Radiotherapy, temozolomide, CCNU, tamoxifen | 35+months                     | Stable 35 months post-resection |
| D2238            | F, 31            | III                | 7              | W            | Radiotherapy, temozolomide, CCNU, tamoxifen | 34+months                     | Stable 34 months post-diagnosis |
| D2239            | M, 56            | IV                 | 7              | W            | Radiotherapy, temozolomide, hydroxyurea, imatinib mesylate | 3 months                     | Died 4 months post-resection |
| D2245            | M, 44            | III                | 9              | W            | Radiotherapy, CCNU, tamoxifen, imatinib mesylate, hydroxyurea, CCNU, bevacizumab, CPT-11 | 18 months                    | Stable 32 months post-resection |
| D2247            | M, 51            | IV                 | 8              | W            | Radiotherapy, temozolomide       | BCNU wafer, O6BG, CPT-11, imatinib mesylate, hydroxyurea | 4 months                  | Died 9 months post-resection |
| D2248            | M, 44            | III                | 6              | W            | Unknown                          | Data not available            | Data not available          |
| D2259            | M, 27            | III                | 7              | W            | Radiotherapy, temozolomide, CCNU | 31+months                     | Stable 31 months post-resection |
| D2261            | M, 52            | III                | 5              | W            | Radiotherapy, temozolomide, CCNU, cloretazine | 7 months                     | Died 10 months post-resection |
| D2262            | F, 40            | III                | 5              | W            | Radiotherapy, temozolomide       | 3 months                      | Unknown                    |
| D2264            | F, 45            | IV                 | 4              | ND           | Radiotherapy, temozolomide, CCNU, CPT-11, tamoxifen | 30+months                    | Stable 30 months post-resection |
| D2268            | M, 59            | IV                 | 6              | M            | Radiotherapy, temozolomide, CCNU, CPT-11, imatinib mesylate, hydroxyurea/PTK787, bevacizumab, carboplatin | 7 months                     | Stable 26 months post-resection |
| D2301            | M, 51            | IV                 | 1              | M            | Nil                              | BCNU wafer, radiotherapy, temozolomide, cilengitide, CCNU, imatinib mesylate, hydroxyurea, PTK787 | 3 months                  | Stable 13 months post-resection |
| D2302            | M, 40            | IV                 | 3              | ND           | Radiotherapy, temozolomide, etoposide, re-resection | 24+months                    | Stable 24 months post-resection |
| LM-G-2           | M, 54            | IV                 | 4              | ND           | Sub-total resection then radiotherapy | 4+months                     | Alive 4 months post-resection |
| LM-G-4           | M, 69            | IV                 | 3              | M            | Gross total resection then radiotherapy | 12 months                    | Alive 4 months post-resection |
| RMH              | M, 74            | IV                 | 3              | M            | Radiotherapy                      | 2 months                      | Alive 4 months post-resection |
| 018              | M, 54            | ex vivo            | ND             | Nil          | Recurrence, resection            | data not available            | Alive 6 months post-resection |
| 019              | M, 62            | ex vivo            | ND             | Nil          | Radiotherapy and temozolomide    | data not available            | Alive 6 months post-resection |
| 020              | F, 52            | ex vivo            | ND             | Nil          | Resections, radiotherapy, temozolomide | data not available            | Alive 6 months post-resection |
| RMH              | M, 66            | ex vivo            | ND             | Nil          | Radiotherapy and temozolomide    | data not available            | Alive 4 months post-resection |
| 021              | F, 54            | ex vivo            | ND             | Nil          | Radiotherapy                      | data not available            | Alive 4 months post-resection |
| RMH              | M, 58            | ex vivo            | ND             | Nil          | Radiotherapy and temozolomide    | data not available            | Alive 4 months post-resection |

HRM = high-resolution melt; M = mutation possibly affecting p53 function; ND = not done; WT = wild type; M<sup>1</sup> = silent mutation; M<sup>2</sup> = mutation predicted by HRM analysis but not identified by sequencing. For details see Supplementary Figure 1. *p53 genotype, as determined by HRM analysis and sequencing.
confluence or maximal confluency for that cell line. Thereafter, cells were expanded into T175 flask (passage 4) for cryostorage, experimentation or propagation as required.

Normal human hepatocytes and astrocytes were purchased from Cambrex (East Rutherford, NJ, USA). The established glioma cell lines D270 and U373 have been characterized previously (Knight et al, 2001, 2004). LN18 cells were purchased from the ATCC (Manassas, VA, USA).

**Drug treatments**

We endeavoured to use physiologically relevant drug concentrations in this study. Cells were exposed to doses corresponding to 100% and 10% of peak plasma or tumour concentrations (Table 2). Data regarding the pharmacokinetics of the various TRAIL formulations in humans have not yet been published. We used soluble TRAIL (Feprotech, Rocky Hill, CT, USA) at concentrations commonly employed in vitro (1 μg ml⁻¹ and 100 ng ml⁻¹). We arbitrarily chose to use F-LZ-TRAIL (Knight et al, 2001) at 10-fold lower doses than soluble TRAIL (100 and 10 ng ml⁻¹), because our previous in vitro analyses showed that it is more potent than the untagged formulation (data not shown). Superkiller TRAIL (Alexis Biochemicals, Lausen, Switzerland) was used at 100 ng ml⁻¹.

**Cell death/survival assays**

During the experiments performed for this study, all cells were cultured in ZO-MEM supplemented with 10% FBS (SAFC Biosciences, Sydney, NSW, Australia). Each sample was analysed in triplicate. High-resolution melt analysis of exons 5 – 8 was used to quantitate survival, according to the manufacturer’s instructions. Five hundred cells were used per treatment. Fifty thousand cells were used per condition for propidium exclusion assays (Knight et al, 2001), which were analysed using an LSRII (BD Biosciences, San Jose, CA, USA).

**Caspase activity assay**

Ten thousand cells were incubated with normal media or TRAIL-based drugs in 96-well plates for 6 h, then caspase (DEVDase) activity was detected using the Caspase-Glo 3/7 kit (Promega), according to the manufacturer’s instructions. Cell-specific luminescence signals were obtained by subtracting the signal generated from plates containing media or drugs but no cells from the signal obtained from wells containing cells and drugs.

**Immunoblotting**

One hundred thousand cells were lysed, subjected to SDS–PAGE, immunoblotted and signals quantitated using previously published protocols (Ashley et al, 2005). The following antibodies were used: rabbit anti-DR4 and anti-DR5 from ProSci (San Diego, CA, USA) (clone 1/FADD), mouse anti-caspase-8 and anti-c-FLIP from Alexis (Lausen, Switzerland) (clones 12F5 and NP6, respectively), mouse anti-FADD from BD Transduction Laboratories (San Jose, CA, USA) (clone 1/FADD), mouse anti-XIAP from MBL (Woburn, MA, USA) (clone 2F1), mouse anti-GAPDH from Chemicon (North Ryde, NSW, Australia) (clone 6C5), rabbit anti-p53 from Cell Signaling (Danvers, MA, USA) (clone 9282), goat anti-mouse-HRP (Sigma, no. A2304) and goat anti-rabbit-HRP (BD Biosciences no. 554021). Control lysates from 293T cells transiently transfected with plasmids directing the expression of caspase-8, FADD, c-FLIPL were generated as previously reported (Ashley et al, 2005). Similar control lysates were made using expression plasmids encoding XIAP, DR4 (kindly provided by Paul Ekert) and DR5. pRES-PL-XIAP was synthesised as follows. Oligonucleotides 1 and 2 were annealed and ligated into pRES-Neo (Clontech, Mountain View, CA, USA) cut with BamHI and NotI, to yield pRES-PL. The coding region of XIAP was amplified with primers 3 and 4, cut with EcoRI and NotI and ligated into EcoRI/NotI cut pRES-PL, generating pRES-PL-XIAP. pRES-Neo-DR5 was made by amplifying the DR5 coding region with primers 5 and 6, cutting with EcoRI and BamHI and ligating into pRES-Neo (Clontech). Oligonucleotides:

1. 5’-GGCGCAATTGCGGCGGATTCGGCGCGCTAAGCAGTCGCGCCGCGAGGCT-3’
2. 5’-GATCCAGGCTTCGGCGGCACCTCATGCTACGGCGCTATGCCGAATTAC-3’
3. 5’-GGAATTCGCGCATGACTTTTAACAGTTTTGAAGG-3’
4. 5’-CCCCCGGGGGTCGAACATACAACTAAATTGTCGTGTCG-3’
5. 5’-GGAATTCGCGCATGAAACCGCGGACAG-3’
6. 5’-GGCGGATCTTGGACATGGCGCGACAGT-3’

**p53 genotyping**

Genomic DNA was extracted from frozen cell pellets (D2234MG, D2235MG, D2238MG, D2245MG, D2247MG, LM-G-4 and LM-G-8) or frozen cell suspensions (D2239MG, D2248MG, D2259MG, D2261MG, D2262MG and D2268MG, D2301MG) using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. PCR cycling and high-resolution melt (HRM) analysis were performed on the Rotor-Gene 6000 (Corbett Research, Sydney, NSW, Australia). Each sample was analysed in triplicate. High-resolution melt analysis of exons 5–8 was performed as described previously (Krypuy et al, 2007). The
amplicon of exon 4 (176 bp) covers the DNA binding domain and
was generated using the primers TP53-Exon4-DBD-F, 5’-
CCCCTGCACCAGCAGCTCCTA-3’ and TP53-Exon4-DBD-R, 5’-
CAGCCCCCTCAGGGCAACTGA-3’. The amplified region corre-
sponds to GenBank accession number AC087388, nucleotides
78962 – 79137. PCR was performed in a 100 µl PCR tube (Corbett
Research) with a final volume of 20 µl, containing 200 nmol l⁻¹
of the forward primer, 300 nmol l⁻¹ of the reverse primer,
200 µmol l⁻¹ of each dNTP, 0.5 U of HotStarTaq DNA Polymerase
(Qiagen) in the supplied PCR buffer containing 2.0 mmol l⁻¹
MgCl₂, 5 µmol l⁻¹ SYTO9 (Invitrogen) and 2.5 ng of genomic DNA
as template. The initial denaturation (95 °C, 15 min) was followed
by 11 cycles of 15 s at 95 °C, 15 s at 65 – 60 °C touchdown (0.5 °C per
cycle), 20 s at 72 °C and 39 cycles of 15 s at 95 °C, 15 s at 60 °C, 20 s at
72 °C; one cycle of 1 min at 95 °C, 72 °C for 1.5 min and a HRM step
from 72 to 95 °C rising at 0.2 °C per second, and holding for 1 s after
each stepwise increment. To confirm the mutation positive HRM
results, PCR products of the entire exon 5 (exons 5a and 5b) and
the HRM products of exons 6 and 8 were purified, directly
sequenced in both directions and analysed as described previously
(Krypuy et al, 2007).

RESULTS

TRAIL sensitivity

Cells from freshly resected gliomas, minimally passaged glioma cell
lines, established glioma cell lines, normal astrocytes and
hepatocytes were exposed to three formulations of TRAIL or an
agonistic antibody, alone or in combination with seven chemothera-
py drugs. Table 1 provides details of the glioma cells used and
the patients from whom they were obtained. The normal cells
tolerated exposure to ‘hepatosafe’ soluble TRAIL, the anti-DR5
antibody and the lower dose of cross-linked TRAIL (Figure 1A).
Higher concentrations of cross-linked TRAIL and superkiller
TRAIL were lethal to both types of normal cells, with hepatocytes
being especially sensitive. As sole agents, the TRAIL formulations
and anti-receptor antibody induced negligible cell death in most of
the glioma samples tested. Only one of the early passage lines,
D2247, was efficiently killed by the two cross-linked formulations
of TRAIL and the anti-DR5 antibody. This line also displayed
intermediate sensitivity to soluble TRAIL. Two other lines, D2234
and D2245, were somewhat sensitive to the cross-linked TRAIL
formulations and the agonistic antibody, but not to soluble TRAIL.
None of the ex vivo samples was substantially sensitive to any of
the TRAIL-based treatments. As reported previously, LN18 and
D270 were TRAIL-sensitive, but U373 was TRAIL-resistant
(Hawkins, 2004). Consistent with the notion that apoptosis was
responsible for the reductions in ATP levels observed in some
drug-treated cells, caspase activity in D2247 cells but not in D2302
cells increased following exposure to TRAIL or anti-DR5
(Figure 1B).

Cooperation between TRAIL and chemotherapy drugs

Previous studies have shown that co-treatment with traditional
anti-cancer agents can sensitize some cells to TRAIL, in vitro and

Figure 1  In vitro responses of glioma cells, astrocytes and hepatocytes to TRAIL. (A) Cells from the indicated early passage or established glioma cell lines, ex vivo gliomas, normal astrocytes or normal hepatocytes were incubated in vitro with TRAIL or with an anti-DR5 antibody. Black triangles indicate high and low
drug concentrations, when applicable (see Table 2 and the Materials and Methods section). Survival was assayed using the CellTiter Glo kit and depicted
using ‘bubble’ graphs. The areas of the circles denote net survival following each treatment, relative to untreated cells (set at 100%, left column). Small circles indicate efficient killing; large circles reflect survival and/or proliferation, as illustrated in the graphical legend. Glioma assays were performed in duplicate (data
are represented by circles). Four replicates were performed for hepatocytes and eight replicates for astrocytes. For astrocyte and hepatocyte data, grey
circles depicting average survival are overlaid upon black circles indicating average survival plus standard error. (B) DEVDase activity in D2247 and D2302
cells was monitored 6 h following treatment with the specified TRAIL formulations, anti-DR5 antibody or normal media.
enhance sensitivity to TRAIL was also explored. Cells from a combination treatments (Figures 2 and 3 and data not shown). Observed when TRAIL-resistant cells were exposed to the TRAIL and anti-DR5 (Figure 2), but only additive toxicity was chemotherapy drugs tested further sensitised D2247 to F-LZ-temozolomide. The other drugs were ineffective as sole agents. All glioma samples. Only the D2235 cells were markedly sensitive to the higher dose of vincristine was weakly toxic to many of the tumours are shown in Figure 3. Many samples were killed by high-dose cisplatin, across all early passage lines and ex vivo one tumour to TRAIL in combination with chemotherapy drugs. Cells from 17 early passage glioma cell lines (A), and 5 uncultured gliomas (B) were incubated in vitro with the stated formulations of TRAIL or anti-DR5 antibody, alone or together with the listed chemotherapy drugs as described in the Materials and Methods section and Table 2. The resulting survival was assayed and graphed as described in the legend to Figure 1. Grey circles depicting average survival are overlaid upon black circles indicating average survival plus standard error.

**Figure 2** In vitro sensitivity of three early passage glioma cell lines and one ex vivo tumour to TRAIL in combination with chemotherapy drugs. Cells from the early passage lines D2235 (A), D2247 (B) and D2248 (C) and the freshly resected glioma RMH020 (D) were incubated in vitro with the stated formulations of TRAIL or anti-DR5 antibody, alone or together with the listed chemotherapy drugs as described in the Materials and Methods section and Table 2. The resulting survival was assayed and graphed as described in the legend to Figure 1.

In general, chemotherapy drugs did not further sensitise D2302 cells to TRAIL. Propidium iodide uptake assays were performed on many of the samples, using selected drug combinations. This method, which gives a direct measure of the proportion of cells killed, yielded similar data to the CellTiter-Glo assay, which quantifies cellular ATP (Figure 4).

**Normal cells**

As previously published (Ashkenazi et al, 1999; Jo et al, 2000; Lawrence et al, 2001; Mori et al, 2004; Ganten et al, 2006), normal human hepatocytes were sensitive to cross-linked TRAIL formulations, but not to soluble untagged TRAIL (Figure 1). Hepatocytes were also efficiently killed by cisplatin and carboplatin (Figure 5). In general, chemotherapy drugs did not further sensitize hepatocytes to TRAIL, although treating hepatocytes with high-dose F-LZ-TRAIL and the chemotherapy drugs elicited a slightly superadditive effect (Figure 5). Normal human astrocytes were also sensitive to cross-linked TRAIL, cisplatin and carboplatin, but not to the same extent as hepatocytes. No significant cooperation in astrocyte lethality was noted between TRAIL and the chemotherapy drugs (Figure 5).

On average, the minimally cultured and uncultured glioma cells were as sensitive or less sensitive than the normal cells to TRAIL-based drugs, alone or in combination with chemotherapeutic agents. However, isolated examples of selective toxicity to glioma cells relative to normal cells were observed (Figure 6). TRAIL formulations combined with platinum-based drugs killed cells
from a few gliomas (D2234, D2247, LM-G-8 and LM-G-2) at least
10 times more efficiently than normal astrocytes and hepatocytes.
Temozolomide cooperated with TRAIL to kill D2235 cells more
efficiently than the normal cells. Some selectivity of TRAIL/
etoposide and TRAIL/vincristine combinations was observed for
D2247 relative to normal cells.

Pathway analyses
To explore potential mechanisms underlying the resistance of most
of the gliomas to TRAIL-induced apoptosis, we surveyed mini-
mally passaged gliomas for the expression of the TRAIL pathway
components DR4 (TRAIL-R1), DR5 (TRAIL-R2), FADD and

Figure 4  Propidium iodide uptake assay of glioma cell sensitivity to
combination treatments. Cells from the indicated early passage glioma
cell lines or ex vivo gliomas were incubated in vitro with soluble TRAIL at
100 ng ml⁻¹ (+) or 1000 ng ml⁻¹ (+ +) alone or together with
temozolomide (13.7 μg ml⁻¹) or cisplatin (54 μg ml⁻¹) for 48 h. Flow
cytometry measurement of propidium iodide exclusion was used to
quantitate the proportion of surviving cells. The areas of the circles denote
survival following each treatment. Small circles indicate efficient killing, large
circles reflect survival.

Figure 5  In vitro sensitivity of astrocytes and hepatocytes to TRAIL in
combination with chemotherapy drugs. Normal human hepatocytes (A)
and astrocytes (B) were incubated in vitro with the stated formulations of
TRAIL or anti-DR5 antibody, alone or together with the listed
chemotherapy drugs. The resulting survival was assayed and graphed as
described in the legend to Figure 1. (A) Quadruplicate assays were
performed to examine hepatocyte survival following incubation with each
TRAIL formulation alone. Grey circles depicting average survival are
overlaid upon black circles indicating average survival plus standard error.
Responses to combination treatment were assayed in duplicate; circles
depict each result. (B) Eight replicates were performed to investigate
astrocyte survival following incubation with each TRAIL formulation alone.
Combination treatments were tested in quadruplicate. Grey circles
depicting average survival are overlaid upon black circles indicating average
survival plus standard error.

Figure 6  Treatments selectively toxic in vitro to glioma cells relative to
normal cells. Coloured circles indicate gliomas killed in vitro by each
treatment at least 3 times (A, C) or 10 times (B, D) more efficiently
than the most sensitive astrocyte replicate (A, B) or hepatocyte replicate
(C, D).
caspase-8, along with potential modulators of TRAIL signalling (cFLIP and XIAP) (Figure 7A–7D). None of the lines expressed detectable DR4 (data not shown) or cFLIP S (Figure 7E). Expression of the other components varied widely between samples. Four of the early passage lines that were TRAIL-resistant (D2259, D2261, D2262 and D2264) did not express detectable FADD. The TRAIL-sensitive line D2247 expressed relatively high levels of DR5 and XIAP and detectable, if relatively low, levels of FADD, caspase-8 and cFLIP L. D2235 and D2301 also expressed low levels of cFLIP L. The p53 status of the majority of lines was also examined.

Figure 7 Immunoblot analyses of candidate TRAIL signalling regulators. Immunoblotting was performed on lysates from the indicated glioma early passage cell lines or 293T cells transiently transfected with expression plasmids encoding the various apoptotic pathway components. (A–D) Anti-DR5 (A), anti-caspase-8 (B), anti-FADD (C) and anti-XIAP (D) signals were quantitated using a chemidoc instrument and plotted relative to the 293T transfectant lysates (set at 100%). A nonspecific band detected by the anti-DR5 antibody is indicated by an asterisk. Long exposures using autoradiograph film revealed some signals too weak to be detected by chemidoc (+). Illustrative immunoblots are inset within each graph. (E) Autoradiography was used to assay cFLIP L and p53 expressions in early passage lines and 293T cells transfected with the cFLIP L expression plasmid (cFLIP L, GAPDH immunoblots) or empty vector (p53 immunoblot). Irrelevant lanes separating the 293T transfectant signals from those of the early passage lines have been removed.
Mutations that may affect function were identified in four of the lines (D2234, D2235, LM-G-4 and LM-G-8) (Supplementary data). D2247 was the only line of those tested to express sufficient p53 to detect by immunoblotting (Figure 7E).

DISCUSSION

Two conditions would have to be met for TRAIL to be clinically effective for treating malignant glioma: (a) a route of administration must be used that delivers concentrations of TRAIL that are lethal to the patient’s glioma cells in vivo and (b) the glioma cells must be marked as being more sensitive to TRAIL than normal cells exposed via that mode of delivery. Any treatment for brain tumours must transverse or bypass the blood–brain barrier. This means that any future TRAIL-based therapies for glioma would probably be administered intracranially. Intracranial delivery may also lessen hepatocyte exposure and thus reduce hepatotoxicity. Multiple intracranial delivery systems are being developed. The post-resection tumour cavity can be lined with drug-impregnated wafers (Westphal et al, 2006). Convection-enhanced delivery is a promising new technique in which drugs are infused at the tumour site under pressure, thus improving distribution into the mass of the tumour (Lopez et al, 2006). Mice bearing intracranial glioma xenografts were successfully treated with TRAIL administered using this approach (Saito et al, 2004).

Numerous studies have concluded that TRAIL is a promising anti-glioma drug based on the investigation of TRAIL sensitivity and signalling in established glioma cell lines. However, it has been reported that melanoma cells exhibited enhanced TRAIL sensitivity following in vitro culture (Nguyen et al, 2001). To maximise the potential for in vitro culturing artefacts, in this study we tested the TRAIL sensitivity of minimally cultured and freshly resected gliomas. Our analyses imply that minimally passaged and uncultured gliomas respond similarly to TRAIL. It is, however, possible that prolonged in vitro culturing, as with the commonly studied established glioma lines, may significantly affect TRAIL sensitivity.

Minimally cultured and uncultured glioma cells were generally resistant to the TRAIL formulations tested. None of the tumours tested was efficiently killed by the ‘hepatosafe’ formulation of TRAIL currently being evaluated in early-phase clinical trials. Three of the gliomas were sensitive to cross-linked formulations of TRAIL: D2234, D2245 and D2247. Intriguingly, the patients from whose tumours those lines were derived had all received chemotherapy before surgery. In contrast, only one patient whose tumour was TRAIL-resistant received treatment prior to resection (RMH020). Unfortunately, normal astrocytes and hepatocytes were also sensitive to cross-linked TRAIL. Only one glioma (D2247) was killed by cross-linked TRAIL more efficiently than normal astrocytes.

Four of the early passage lines lacked detectable FADD, possibly accounting for their TRAIL resistance. The TRAIL-sensitive line D2247 bore only low levels of FADD and caspase-8, arguing that low concentrations of these pathway components can be sufficient for TRAIL-induced apoptotic signalling. D2247 was one of a number of lines lacking mutation in p53, and the only line tested to express p53 levels detectable by immunoblotting. Consistent with this observation, D2247 also expressed high levels of DR5, a known p53-inducible protein (Wu et al, 1997). LM-G-4, a TRAIL resistant line, expressed higher levels of DR5, caspase-8 and FADD than D2247, arguing that factors other than the levels of these proteins influence TRAIL sensitivity in glioma cells. The expression of the potential TRAIL inhibitors XIAP and cFLIP was higher in the TRAIL-sensitive D2247 cells than the resistant lines, arguing against overexpression of these proteins as a mechanism of resistance in gliomas that survived incubation with TRAIL. Definition of the molecular mechanisms contributing to the resistance of the majority of the gliomas to TRAIL will require additional investigation, but it seems unlikely that a single resistance mechanism, perhaps amenable to therapeutic manipulation, will be found to account for the widespread survival of glioma cells following exposure to TRAIL.

Better therapies are urgently needed for malignant glioma. In vitro sensitivity assays could be used for preclinical evaluation of the anti-glioma potential of new drugs. In the future, such assays could also assist in the selection of the most effective drug combinations for individual patients. Our data imply that few glioma patients would benefit from TRAIL-based therapies; perhaps in vitro sensitivity testing could help identify the minority of patients most likely to respond. The luminescence assay used in this study is rapid, high-throughput and requires fewer cells than the commonly employed MTT and flow cytometric assays, thus allowing more drug doses and combinations to be tested per sample. The accuracy with which in vitro sensitivity testing predicts in vivo responses may be influenced by the mechanism of action of the particular drug as well as the degree to which cellular environment influences the toxicity of each agent to glioma cells. For example, glioma cell interactions with surrounding cells and the extracellular matrix could modulate the apoptosis-inducing capacity of anti-cancer drugs in vivo. For these reasons, it is also important to examine orthotopic models of cancer. Notwithstanding these considerations, in vitro apoptosis data do tend to correlate with patient responses in cancer types for which truly effective drugs exist (Nagourney, 2006). The dearth of effective drugs for treating glioma has limited assessment of the predictive value of in vitro sensitivity testing and toxicity tumour assays. Nevertheless, available evidence does argue that in vitro sensitivity testing can assist in selecting treatments for glioblastoma patients (Iwadate et al, 2003).

Currently, the majority of glioblastoma patients receive surgical resection, radiotherapy and temozolomide. Although temozolomide may benefit some patients, it is well recognised that the majority of patients do not respond to this drug. Temozolomide administration only extended median survival of glioblastoma patients from 12.1 to 14.6 months post-diagnosis, boosting 2-year survival from 10.4 to 26.5% (Stupp et al, 2005). Given this subtle effect in vivo, it is perhaps not surprising that only one of the tumours tested in this study (D2235) was efficiently killed by temozolomide in vitro. Cisplatin was the most effective of the drugs tested in vitro, however, sensitivity was triggered only by the higher of the doses employed, which corresponded to the peak intratumoral cisplatin level following embolisation (Tegeder et al, 2003). Only partial sensitivity was observed using a 10-fold lower concentration, which resembles peak tumour and plasma levels achieved following systemic cisplatin administration (Riva et al, 2000; Tegeder et al, 2003; Watanabe et al, 2003; Siegel-Lakhai et al, 2005). A few gliomas were more sensitive than normal cells to treatments with TRAIL and chemotherapy drugs.

In conclusion, our data indicate that TRAIL-based therapies would be unlikely to benefit the majority of glioma patients. This study does, however, suggest that particular patients may respond to specific combinations of TRAIL and chemotherapy drugs. In vitro sensitivity assays may prove useful in identifying such patients and predicting effective drug combinations.

ACKNOWLEDGEMENTS

We are very grateful to Stan Styli for help with tumour acquisition, to Sarah Roberts for technical assistance and to John Silke and Don Phillips for providing reagents. This study was funded by grants from the Cancer Council Victoria (to CJH, DMA and HSP), Cure-for-Life Foundation (to CJH) and ANZ Trustees (to CJH).

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)
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TRAIL sensitivity of ex vivo and early passage gliomas

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British Journal of Cancer (2008) 99(2), 294 – 304 © 2008 Cancer Research UK