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The induction of myeloma cell death and DNA damage by tetrac, a thyroid hormone derivative

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Abstract

Multiple myeloma (MM) is a plasma cell malignancy in which involvement of the thyroid hormone-integrin αvβ3 pathway was shown, and pharmacologic inhibition of this pathway is a rational approach to disease management. A thyroid hormone derivative, tetradiothyroacetic acid (tetrac), which inhibits l-thyroxine (T4) and 3,5,3′-triiodo-l-thyronine (T3) binding to αvβ3 integrin, was studied in five MM cell lines and primary bone marrow (BM) MM cells. Tetrac inhibited MM cell proliferation (absolute cell number/viability) and induced caspase-dependent apoptosis (annexin-V/PI and cell cycle). Activation of caspase-9 and caspase-3 was further demonstrated. Moreover, DNA damage markers, ataxia-telangiectasia-mutated (ATM) kinase, poly ADP-ribose polymerase (PARP-1) and histone γH2AX were induced by tetrac. The various tetrac-initiated effects were attenuated by Arg-Gly-Asp (RGD) peptide, suggesting integrin involvement. Primary BM mononuclear cells were harvested from MM patients (n = 39) at various disease stages. Tetrac-induced apoptosis (12/17 samples) and sensitized the cytotoxic action of bortezomib (6/9 samples). Lastly, expression of plasma membrane integrin αvβ3 was shown not only in the malignant plasma clone, but also in other cell populations within the BM samples (n = 25). Tetrac is anti-proliferative and pro-apoptotic in MM and cells may offer a therapeutic approach for this disease.

Introduction

A large number of epidemiological and human studies have demonstrated an association between thyroid hormone and cancer. In patients with elevations of serum thyroid hormone, an increased risk of cancer compared with euthyroid patients has been reported (Ness et al. 2000, Hellevik et al. 2009). An association between thyroid dysfunction and the risk of development of multiple myeloma (MM), a plasma cell (PC) malignancy accounting for more than 13% of hematological malignancies, has also been suggested (Dalamaga et al. 2008). Another body of epidemiological and clinical evidence suggests improved survival in individuals with a variety of cancers who also have hypothyroidism (Cristofanilli et al. 2005, Nelson et al. 2006, Illouz et al. 2009, Baldazzi et al. 2010, Hercbergs et al. 2010, Schmidinger et al. 2010). A few years ago, a binding site for the thyroid hormones
1-thyroxine (T₃) and 3,5,3′-triiodo-1-thyronine (T₂) was described on αvβ3 integrin (Bergh et al. 2005). This integrin is overexpressed in an array of cancer types (Desgrozelle & Cheresh 2010), including MM (Vacca et al. 2001, Ria et al. 2002, Tucci et al. 2009). The affinity of the receptor is higher for T₄ than for T₃ (Bergh et al. 2005), and binding of T₄ occurs at physiological free T₄ levels. The cell surface receptor-binding site for thyroid hormones on integrin αvβ3 is located in close proximity to the well-investigated Arg-Gly-Asp (RGD) recognition site. This region of αvβ3 contains receptors for other small molecules in addition to thyroid hormones that do not contain an RGD sequence and thus are ‘non-RGD’ binding sites. This thyroid hormone-binding site has been studied by crystallography and mathematical modeling of the thyroid hormone-binding kinetics (Aghajanova et al. 2011). The site has no structural homologies with the nuclear thyroid hormone receptors (TRs) that mediate genomic actions of the hormone. The description of a cell surface receptor binding site that is unrelated to conventional nuclear TRs has served to define the contributions of non-genomic actions of thyroid hormones to cancer. Upon binding of either hormone to the integrin, numerous effects are exerted by activating several signaling pathways (Davis et al. 2011). Although the thyroid hormone-integrin axis has been shown to be present in an array of tumor types, our group suggested a role for this pathway in a hematological malignancy, MM. This was confirmed by showing an increase in cell proliferation and activation of MAPK by T₃/T₄ via the αvβ3 integrin (Cohen et al. 2011). In this current work, we have characterized the effects of an antagonist at the hormone receptor site on the integrin, tetraiodothyroacetic acid (tetrac). Tetrac is a naturally occurring thyroid hormone derivative with low-potency thyromimetic activity inside the cell at TRs, but that selectively blocks binding of T₄ and T₃ to the thyroid hormone receptor site on αvβ3 (Davis et al. 2011, 2016). This agent was shown by in vitro and animal models to reduce cancer cell proliferation, cell migration and angiogenesis (Mousa et al. 2008, 2012, Rebbaa et al. 2008, Yalcin et al. 2009), to induce apoptosis in tumor cells (Glinskii et al. 2009, Yalcin et al. 2010) and to foster DNA double-strand breaks (Hercbergs et al. 2011). Tetrac has also been shown to chemosensitize several cancer cells that express chemoresistance (Rebbaa et al. 2008). Despite this extensive research in solid tumor models, tetrac has never been studied as a potential therapeutic agent in MM.

Methods and materials

Cell lines and primary BM cells

MM cell lines: CAG and ARK (established at the Arkansas Cancer Research Center), RPMI-8226 (CCL 155, ATCC), U266 (TIB-196, ATCC) and ARP-1. All cell lines were negative for mycoplasma contamination. Bone marrow (BM) aspirates were obtained upon written consent from a total of 40 patients at various disease stages (clinical data are presented in Supplementary Table 1, see section on supplementary data given at the end of this article). Additionally, one normal BM sample from healthy subject was collected. All BM aspirates were obtained from patients treated at the Meer Medical Center after approval by an Institutional Review Board (IRB number #180-2010). BM mononuclear cells were isolated by Ficoll-Paque gradient centrifugation according to the manufacturer’s instructions (Sigma-Aldrich). The different mononuclear cell populations were characterized and immunophenotyping was performed by flow-cytometry. Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, penicillin and streptomycin antibiotics and the passage number for the experiments described was up to 20.

Reagents and chemicals

T₃, T₄ and tetrac (Sigma-Aldrich) were dissolved in DMSO to 100 mM and further dissolved in 1 mM KOH-propylene glycol, which was also used as a vehicle control (final concentration of 0.04 N KOH with 0.4% polyethylene glycol, which was also used as a vehicle control (vol/vol)). RGD and Arg-Gly-Glu (RGE) peptides (Sigma-Aldrich) were dissolved at 100 mM in PBS. Bortezomib (dissolved in saline to 2.6 µM) was obtained from the oncology pharmacy at Meer Medical Center. The volume of solvent added to each well has been kept constant for all conditions. Pan-caspase inhibitor, Z-VAD-FMK was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Monoclonal antibody against αvβ3 integrin (LM609, PE/FITC-conjugated) was purchased from Chemicon International. APC-CD138 antibodies were from Miltenyi Biotec (Bergisch Gladbach, Germany), FITC-CD45 and PE-CD38 antibodies were from BioLegend, San Diego, CA, USA. Proliferating cell nuclear antigen (PCNA) antibody was from Santa Cruz Biotechnology. Antibodies against total and cleaved caspase-9, caspase-3, PARP-1 and tubulin were from Cell Signaling. Anti-AIF was from EMD Millipore. Anti-pATM (phospho-serine 1981) was from Epitomics (Burlingame, CA, USA) and anti-pH2AX
(phospho-serine 139) from Chemicon International. All antibodies were appropriately validated.

**WST-1 proliferation assay**

WST-1 reagent (Roche; 10% final concentration) was incubated according to the manufacturer's instructions as previously described (Cohen et al. 2015).

**Flow-cytometry**

Absolute cell number: cells were harvested in a fixed volume and counted. Cell cycle: following permeabilization and fixation by incubation with 70% ethanol (20 min, −20°C), the cells were stained with DNA propidium iodide (PI) (50 µg/mL) for 15 min at room temperature, along with RNase A (10 µg/mL) (Sigma-Aldrich) and cell cycle analysis was carried out by flow-cytometry (MACSQuant, Miltenyi Biotec). Apoptosis/necrosis: apoptotic cell death was measured by annexin-V/PI assay (BioVision) as previously described (Shinderman-Maman et al. 2016). Immunophenotype: immunophenotyping was performed in BM-derived mononuclear cells by using FITC-CD45, PE-CD38 (data not shown) and APC-CD138 antibodies. αvβ3 expression: cell lines and primary BM cells were harvested in RPMI-1640 medium and labeled with 10 µg/mL FITC-αvβ3 or PE-αvβ3 antibody (LM609).

**RNA extraction and cDNA synthesis**

RNA was extracted using a Nucleospin RNA II kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions and eluted in 40 µL RNase-free water, as previously described (Cohen et al. 2015). RNA concentration and purity were measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). The proteins (30 µg) were separated on 10–12.5% polyacrylamide gels, transferred to PVDF membranes and analyzed by Western blot, using primary antibodies and appropriate second HRP-conjugated antibody (Jackson Immuno-Research Laboratories). Immunoreactive proteins were detected by chemiluminescence reagents (Biological Industries, Beit Haemek, Israel). Alpha tubulin or beta actin was used for normalization. Band intensity was visualized using LAS-3000 (FujiFilm, Tokyo, Japan) and quantified by Multi-gauge, v3.0 software and normalized to protein amount.

**Real-time PCR**

Complementary DNA (cDNA) was analyzed for the following apoptotic genes: Apoptotic protease activating factor-1 (apaf1, accession number Nm_013229), caspase-3 (accession number Nm_004346), p53-upregulated modulator of apoptosis (PUMA, accession number Nm_014417), NOXA (accession number Nm_021127) and FAS (accession number Nm_000043), by 7500 Fast Real-Time PCR system (Applied Biosystems), using Applied Biosystems Fast Sybr Green Mix (cat # 4385612).

Results, normalized to beta actin (accession number Nm_001101), were calculated as -fold change, using the comparative threshold cycle method (2−ΔΔCT) relative to control cells (i.e., controls were assigned a value of 1 by definition). Primers (Hylabs, Rehovot, Israel) were designed as published before (Cohen et al. 2011) in different exons in order to eliminate DNA contamination, using Primer-Express software (Applied Biosystems). Different bioinformatics tools, including BLAST and alignment PCR analysis, were used to design primers suitable for the amplification of fragments from the candidate genes. Primers sets were Apaf1: F TGCGCTGCTCTGCTTTCT, R: CCATGGTGAGCAGCTTTCT. Caspase 3: F: CAGACAGTGGTGATGATGAC, R: TCGCCAAATATAAACCGTG. Puma: F: GAGAGCAAATGAGCCAAACG, R: GGAGCAACCGGCAACAG. Noxa: F: CCGAGATGCGTTGGAAGAA, R: CCAAATCTCCTGAGTTGAGTACA. Fas: F: CCCTCTACCTCTGGTGTTTAC, R: TGATGTCAGTCATCGGAC. Beta actin: F: CCTGGCAACCGACACAAT, R: GCGATCCACAGGAGTACT.

**Western blotting**

Equal numbers of cells (10⁶ cells/6-well plate) were used for total protein extraction quantified by BCA Protein assay (Thermo Scientific). The proteins (30 µg) were separated on 10–12.5% polyacrylamide gels, transferred to PVDF membranes and analyzed by Western blot, using primary antibodies and appropriate second HRP-conjugated antibody (Jackson Immuno-Research Laboratories). Immunoreactive proteins were detected by chemiluminescence reagents (Biological Industries, Beit Haemek, Israel). Alpha tubulin or beta actin was used for normalization. Band intensity was visualized using LAS-3000 (FujiFilm, Tokyo, Japan) and quantified by Multi-gauge, v3.0 software and normalized to protein amount.

**Inhibition of caspase-dependent apoptosis**

MM cells were treated in the presence/absence of 50 µM pan-caspase inhibitor, Z-VAD-FMK, dissolved in DMSO.

**Statistical analysis**

Mean ± s.d. was calculated for each value. In order to compare the means for pairs of groups (treatment vs control), Student’s unpaired t-test was used. A value of P < 0.05 was considered significant.
Results

Tetrac inhibits MM cell proliferation and viability and induces apoptosis

We have previously demonstrated in MM cell lines that the activation of pERK by the thyroid hormones is effectively inhibited by tetrac (Cohen et al. 2011). Additional experiments indicated that the reduction of apoptotic genes, mainly by T₄, was reversed in the presence of tetrac (Supplementary Fig. 1). These anti-mitogenic actions were further explored in four MM cell lines (U266, ARK, RPMI-8226 and CAG). The cells were seeded (100,000/96-well plate) and treated daily with increasing concentrations of tetrac (10, 50 and 100 µM) for 24–96 h. At each time point, cell proliferation was estimated by WST-1 proliferation assay (Fig. 1A) and cell counts (Fig. 1B). Tetrac reduced MM cell proliferation by 50–75% in all cell lines tested, in a time/dose manner that was cell specific. In parallel, a reduction in the absolute cell number, starting at 48 h, was documented in all of the cell types.

Apoptosis analysis (annexin-V/PI, Fig. 1C) further indicated a time-dependent reduction in cell survival (An−/PI−) with a maximal effect after 72 h of 50µM tetrac (35–63%) and 100µM tetrac (76–90%). In parallel, an increase in apoptosis in response to 50–100µM tetrac for 24–96 h was shown (Fig. 1D). The percentage of cells in early apoptosis (An+/PI−) and late apoptosis/necrosis (An+/PI+, An−/PI+) are presented as % of apoptotic cells. Representative annexin-V/PI results for RPMI-8226 cells and CAG are depicted in Supplementary Fig. 2A and B, respectively. Induction of cell death was further shown by cell cycle analysis (Fig. 1E). A representative cell cycle histogram for RPMI-8226 cells is presented in Supplementary Fig. 2C.

Tetrac activates caspases, but not AIF, in MM cell lines

To assess whether the induction in apoptosis involved the activation of caspases, RPMI-8226 cells (10⁶ cells/6-well plate) were treated with increasing tetrac concentrations (1–50 µM) for 1–8 h. Doxorubicin (1 µM) served as a positive control. Cleavage of caspase-3, caspase-9 and PARP-1, normalized to the non-cleaved form, as well as AIF levels, were analyzed by Western blots. In details, representative blots (Fig. 2A) indicate that cleavage of caspase-9 was induced following 1–4 h of incubation with 1 µM tetrac, as well as after 8 h in the presence of 50 µM tetrac concentration. Caspase-3 cleavage was completely absent at 1–2 h, while following an incubation of 4 h with 1 µM tetrac, an induction was observed. This effect was sustained at 8 h of treatment at all concentrations examined. No significant effect on PARP-1 level was observed. Results in additional four MM cell lines are presented in Supplementary Fig. 3. The levels of apoptosis-inducing factor (AIF), a caspase-independent apoptosis marker, were unchanged (Fig. 2B). The calculated results following tetrac incubations are presented in Fig. 2C.

Characterizing involvement of p53 and caspases in action of tetrac in MM

After observing that caspases were induced by tetrac, we determined whether tetrac activities were caspase dependent, using a pan-caspase inhibitor, Z-VAD-FMK. In addition, p53 involvement was studied using RPMI-8226 p53-temperature-sensitive cells, which express wild-type p53 at permissive (32°C) and mutant p53 (E285K) at restrictive (37°C) temperatures (Teoh et al. 2000, Hu et al. 2015). The cells were treated with tetrac (50 µM) in the presence/absence of Z-VAD-FMK (50 µM) at 32°C and 37°C for 96 h and were first assessed for cell proliferation by WST-1 (Fig. 3A and B, black bars) by about 0.5-fold. This effect was not reversed by Z-VAD-FMK. Similar results (Fig. 3A and B, gray bars) were observed under permissive temperature (32°C, wild-type p53). When comparing the effect of tetrac on cell proliferation under both p53 conditions it appeared that tetrac’s effect was more significant in the absence of wild-type p53 (Fig. 3A, marked by #). This effect was not reversed by Z-VAD-FMK in both permissive (32°C, wild-type p53) and restrictive (37°C, mutant p53) temperatures.

Next, cell death was determined by performing annexin-V/PI and cell cycle assays (flow-cytometry). Results (Fig. 3C) indicated that in cells bearing mutant p53 (37°C, black bars), as well as in cells containing wild-type p53 (32°C, gray bars), tetrac induced about a 3-fold increase in apoptotic cell death. A comparison of the apoptotic effect of tetrac between the two temperatures, depicted a greater significant effect in p53-independent cells (Fig. 3C, marked by #). Induction of cell death was partially inhibited by Z-VAD-FMK. Representative annexin-V/PI results for RPMI-8226 cells are depicted in Supplementary Fig. 4A.

Cell cycle analysis indicate that in cells lacking wild-type p53 (37°C, Fig. 3D), a potent increase in SubG1 was obtained with tetrac and was reversed by Z-VAD-FMK. However, in cells containing wild-type p53 (32°C, Fig. 3E), a parallel increase in S-G2M arrest was
Tetrac increases DNA damage response markers in MM cells

Many cytotoxic antagonists lead to cell death via their effect on DNA damage response (DDR). We evaluated by Western blotting the effect of tetrac on levels of three central DDR protein markers, phosphorylated ataxia-telangiectasia mutated (pATM), PARP-1 and histone γH2AX, in RPMI-8226 cells treated with increasing concentrations of tetrac (1–50 µM). Doxorubicin served as a positive control. Representative blots (Fig. 4) indicate a rapid and dose-dependent activation of pATM following 1 and 4 h of treatment, while at 2 h or 8 h, this effect was absent or greatly

Figure 1

Tetrac inhibits MM cell proliferation and viability and induces apoptosis. Four MM cell lines were treated with increasing concentrations of tetrac (10–100 µM) for 24–96 h and evaluated for (A) Cell proliferation (WST-1, ELISA), (B) cell number (flow-cytometry), (C and D) apoptosis (Annexin-V/PI) and (E) cell cycle, by flow-cytometry. Experiments were repeated three times in triplicate. *P ≤ 0.05, **P ≤ 0.01.
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Attenuated. PARP-1 was induced by all tetrac concentrations after 4 h of treatment, while no significant effect was observed at the remaining times. Regarding γH2AX, activation was evident after 1 h of treatment with 10 and 50 µM tetrac. At 2 h of treatment, an increase was observed for 1 and 10 µM tetrac concentrations. γH2AX activation by 1 µM tetrac was sustained after 4 h of treatment. A second peak in γH2AX phosphorylation was observed after 8 h of treatment, with 10 and 50 µM tetrac concentrations. The calculated results following tetrac incubations are presented in Fig. 4B. Supplementary Figure 5A, B and C depicts results in three additional MM cell lines, presenting cell-specific time- and dose-dependent response to tetrac.

The effects of tetrac on proliferation, viability, apoptosis and DNA damage repair in MM cells are blocked by RGD

Tetrac-binding site on integrin αvβ3 is at the thyroid hormone receptor site, near the RGD-recognition site (Cody et al. 2007, Freindorf et al. 2012, Davis et al. 2016). To support integrin involvement, RGD peptide was studied for its effects on tetrac actions. CAG cells were seeded (100,000/96-well plate) and treated in the presence/absence of increasing concentrations of RGD peptide (50, 100 or 200 µM) and tetrac (25–100 µM) overnight (24 h of treatment). Representative cell viability experiment indicates that high concentrations of RGD, alone (Fig. 5A, gray line), did not affect cell proliferation, while tetrac, alone (Fig. 5A, black line), reduced cell proliferation in a concentration-dependent manner. This effect was allosterically interfered by increasing RGD concentrations (Fig. 5A, dashed gray lines). No effect was observed with the negative control RGE peptide (Fig. 5B). Comparable viability results were obtained in RPMI-8226 cells (Supplementary Fig. 4D). In addition, cell death (annexin-V/PI) was explored in CAG cells (100,000/96-well plate) treated overnight with high tetrac concentration (100 µM) in the presence or absence of RGD (1–500 µM). Representative results (Fig. 5C) demonstrate that tetrac reduced the surviving fraction (An−/PI−) by about 50%, and this effect was hindered by RGD. In accord with the proliferation results, RGD alone had no effect. The increase in apoptotic cell death in response to 100 µM tetrac was effectively inhibited by RGD (Fig. 5D). Representative annexin-V/PI results are depicted in Supplementary Fig. 4C.

We next examined whether RGD interfered with the action of tetrac on DNA damage/repair. CAG cells

Figure 2
Tetrac activates caspases and PARP-1 cleavage, but not AIF in MM. RPMI-8226 cells were incubated with tetrac (1–50 µM) for 1–8 h, total protein was extracted and Western blots were carried out for (A) caspase-9, caspase-3, PARP-1 and (B) AIF. Shown is a representative experiment of two performed (C) Representative quantification of band intensity of the corresponding Western blots is presented as ratio of cleaved target protein over total protein intensity. Values are means ± s.d. Densitometry is expressed as percentage compared with the vehicle-treated group (considered as 100%, marked by a dashed line). Experiments were repeated twice. *P ≤ 0.05, **P ≤ 0.01.
were incubated for 24h with increasing concentrations (0.1–100µM) of tetrac and RGD, together and alone (Fig. 5E). A representative blot indicates that tetrac significantly increased the level of γH2AX phosphorylation by 2.8–4.67 fold, while RGD alone had no effect. When 1µM tetrac was combined with RGD (0.1–100µM), the induction in γH2AX phosphorylation by tetrac was completely prevented (Fig. 5E, gray bars). Thus, the cancer cell-killing actions of tetrac are initiated at the integrin and the RGD recognition site and the tetrac-thyroid hormone-binding site on αvβ3 are proximal to one another.

Tetrac induces apoptosis in primary BM cells from MM patients

After establishing the cytocidal effects of tetrac in MM cell lines, we examined the effect of the drug on primary BM cells. Mononuclear cells were isolated from the bone marrow of a myeloma patient (BM #1). The cells were seeded (100,000 cells/96 wells), treated in triplicates with tetrac (100nM or 1µM) for 96h and tested by annexin-V/PI assay for the presence of early apoptosis (an+/-PI−) and for late apoptosis/necrosis (an+/PI+, an−/PI+) by flow-cytometry. Samples of the same cells treated with vehicle served as controls. Results demonstrate the induction of apoptosis by tetrac in whole mononuclear cell population (Fig. 6A) and in gated CD-138+ plasma cells (Fig. 6B), in comparison to untreated cells. Following this observation, the experiment was repeated with tetrac for 24–96h in an additional sixteen mononuclear BM samples (BM #1-17; clinical features are presented in Supplementary Table 1). The results from the entire study cohort (a total of seventeen samples) are presented in Supplementary Table 2. An induction in apoptotic cell death, in response
to low tetrac concentrations, was observed in twelve samples in comparison to samples of the same subject treated with vehicle (considered as 100%), in which the mean rate of basal % of apoptosis was 15 ± 5.

**Tetrac sensitizes MM cell lines and primary BM cells to the cytotoxic effect of bortezomib**

Bortezomib is the first proteasome inhibitor in clinical use and is a leading treatment agent in MM (Chen et al. 2011). Based on the observation that thyroid hormone can antagonize bortezomib’s action (Cohen et al. 2015), together with the anticancer activity of tetrac, led us to explore the potential of combined tetrac-bortezomib treatment. RPMI-8226 and CAG cells were seeded (100,000/96-well plate), treated overnight with tetrac (100 µM) with or without bortezomib (25 nM). Results indicate that bortezomib alone significantly decreased cell proliferation in RPMI-8226 cells by 68% and in CAG cells by 80%; tetrac results were by 60% and 72% reductions, respectively (Fig. 7A). The combined treatment lowered the proliferation rate by 80% in RPMI-8226 and by 90% in CAG cells. Cell survival (annexin-V/PI, Fig. 7B) was reduced by bortezomib in RPMI-8226 by 35% and in CAG cells by 70% while with tetrac by 35% and 50%, respectively. The combination of the two agents resulted in 55% and 85% reductions in cell survival in the two cell lines, respectively. We then evaluated this treatment approach in primary BM mononuclear cells from a representative MM patient (BM#1) treated with bortezomib (25 nM) with/without tetrac (100nM–1 µM). Tetrac sensitized the cells to the cytotoxic effect of bortezomib in the mononuclear cell population (Fig. 7C), and this sensitization was also demonstrated in CD138+ plasma cells (Fig. 7D). A representative annexin-V/PI assay is presented for whole BM cells (Fig. 7E, upper panel) and for CD138+ cells (Fig. 7E, lower panel). The same experiment was conducted on a total of eight additional BM samples, with comparable results obtained in five samples (BM#3-4, 7, 10 and 11), while in three samples (BM#2, 8 and 9), no sensitization was achieved (data not shown).

**Primary BM cells from MM patients express αvβ3 integrin**

The pleotropic actions of thyroid hormone have been shown to be αvβ3 integrin mediated in myeloma cell lines (Cohen et al. 2011) and in mononuclear cells from the BM of myeloma patients (Cohen et al. 2015). αvβ3 integrin expression in myeloma cell lines, composed of 100% plasma cells (CD138+), is depicted in Supplemental Fig. 6. Comparable expression was shown on sorted plasma cells from BM of myeloma patients.
patients (Ria et al. 2002, Tucci et al. 2009, 2016). Our experimental model utilizes unsorted primary BM plasma cells, allowing study of the malignant clone within its natural surrounding cellular niche. We aimed to establish the integrin expression in this experimental cellular platform.

Mononuclear cells from the BM of a myeloma patient (BM#17, relevant clinical data is depicted in Supplementary Table 1) were stained with a FITC-labeled αvβ3 antibody. Flow-cytometry analysis (Fig. 8A) depicts a high expression of αvβ3 integrin in 82% of the cells. We then determined whether the high integrin expression is exclusively contributed by the malignant plasma cell clone. For that, the cells were stained with a plasma cell marker (CD138-APC) and a general mononuclear cells marker (CD45-FITC). Figure 8B depicts the malignant plasma clone (CD138+/CD45-, pink color) and the non-malignant mononuclear cells (CD138-/CD45+, green color). Next, the cells were stained with αvβ3-FITC antibody and gated for the previously described cell populations. Figure 8C indicates that the malignant plasma cells (pink color) highly express this integrin, but this expression is also evident in non-malignant mononuclear cells (in green). Forward scatter (FSC) and side scatter (SSC) plot of the various cell populations is presented in Fig. 8D. The experiment was repeated twice. Representative quantification of band intensity of the corresponding Western blots is presented as ratio of target protein to tubulin intensity. Values are means ± s.d. Densitometry is expressed as percentage compared with the vehicle-treated group (considered as 100%, marked by a dashed line). *P ≤ 0.05, **P ≤ 0.01.
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Discussion

Tetrac is a naturally occurring deaminated analog of T4 that binds the αβ3 integrin in two orientations and competitively displace both T3 and T4, eliminating their non-genomic activities (Bergh et al. 2005, Davis et al. 2011, Freindorf et al. 2012). Tetrac possesses low hormone activity because of shortening of the side chain on the inner ring (removal of a carbon and amine), resulting in the conversion of propionic acid (thyroid hormone) to acetic acid (tetrac/triac). This transforms the compounds from thyroid agonist at the thyroid hormone receptors, to antagonist (Davis et al. 2011). These antagonistic effects were extensively studied in vitro and in animal cancer models (reviewed in Davis et al. 2016), but never examined in MM. In the current work, we have focused on blocking the thyroid hormone-αβ3 axis using tetrac in αβ3 integrin expressing MM cells. By a number of complementary methods in several myeloma cell lines, tetrac produced anti-proliferative effects that were not affected by caspase inhibition and pro-apoptotic effects that were caspase-9 and caspase-3-dependent. No involvement of AIF, a caspase-independent apoptosis marker, was recognized. The peak antitumor action of unmodified tetrac nongenomically initiated at the thyroid hormone-tetrac receptor site on integrin αβ3 is at 10−7-10−6 M (Lin et al. 2011). The antagonistic effects were observed by tetrac in the myeloma cell models mainly at high molar concentrations. However, no toxicity was detected in mice treated with up to 60mg/kg tetrac (equivalent to 250µM tetrac concentration) (Rebbaa et al. 2008). At the indicated concentrations, tetrac was well tolerated, and no significant toxicity was noticed, including unchanged animal weights over the course of the study. To note, the effects produced by tetrac were not always concentration dependent. Similar non-linear integrin-ligand association with other ligands (activators/inhibitors) was reported (Legler et al. 2001, Li & Springer 2017). This may be attributed to the fact that integrins are altered following binding of ligands (Campbell & Humphries 2011). Such changes in the integrin configuration may alter the accessibility of ligands at different concentrations to various receptor sites on the integrin. Tetrac binding may therefore alter the integrin conformation, producing a non-linear effect.

To study the role of p53, a central regulator of apoptosis, in tetrac’s pro-apoptotic action, temperature-sensitive RPMI-8226 cells that express mutant p53

Figure 6

Tetrac induces apoptosis in primary BM cells from MM patients. (A) Mononuclear cells from a MM BM sample (BM#1) were separated by Ficoll gradient, treated in triplicates with tetrac (100nM–1µM) for 96h and then analyzed for cell death (Annexin-V/PI, flow-cytometry). (A) Representative results for whole BM cells and (B) CD138+ cells. Samples of the same subject treated with vehicle served as controls. A full color version of this figure is available at https://doi.org/10.1530/ERC-17-0246.
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In the absence of active p53, tetrac induced caspase-dependent apoptosis, while in the presence of wild-type p53, the induction of caspase-dependent cell death was accompanied by cell cycle arrest that correlates with the well-established role of p53 as a cell cycle regulator (Giono & Manfredi 2006).

Figure 7
Tetrac sensitizes MM cell lines and primary cells to bortezomib. RPMI-8226 and CAG cells were treated with bortezomib (25 nM) with/without tetrac (100 µM) and were analyzed for (A) cell proliferation (WST-1, ELISA) and (B) survival (annexin-V/PI, flow-cytometry). Experiments were repeated at least 3 times, in triplicate. Primary BM cells from MM patients were treated with bortezomib (25 nM) with/without tetrac (100 nM–1 µM) and were analyzed for (C and D) apoptosis (annexin-V/PI, flow-cytometry). (E) Representative annexin/PI results from a BM sample (BM#1) in whole BM cells (upper panel) and gated CD138+ plasma cells (lower panel). *P ≤ 0.05, **P ≤ 0.01. A full color version of this figure is available at https://doi.org/10.1530/ERC-17-0246.

(E285K) at 37°C and wild type at 32°C, were used (Teoh et al. 2000, Hu et al. 2015). In the absence of active p53, tetrac induced caspase-dependent apoptosis, while in the presence of wild-type p53, the induction of caspase-dependent cell death was accompanied by cell cycle arrest that correlates with the well-established role of p53 as a cell cycle regulator (Giono & Manfredi 2006).

Key proteins that coordinate the recognition of DNA damage, ATM, PARP-1 and histone γH2AX were rapidly induced by tetrac. In cell-free assays (unpublished data), we have demonstrated that tetrac does not directly bind to DNA and induce breaks, but rather inhibits DNA repair mechanisms. This observation is supported by experiments in which this drug did not induce direct DNA damage but led to a potent reduction in repair mechanisms (Hercbergs et al. 2011, Leith et al. 2017). Additional experiments, such as ChIPSeq, should be performed to reveal the mechanism by which tetrac inhibits DNA repair. To note, unmodified tetrac may also interfere with the classical hormones action at other levels, such as the cellular entry of T4 and other non-genomic effects that do not involve αβ3 signaling. Therefore, additional intracellular effects by unmodified tetrac cannot be ruled out.
We have reported that thyroid hormone antagonizes the cytotoxic effect of bortezomib (Cohen et al. 2015). Given that tetrac is a selective inhibitor of the T$_{i}$/T$_{v}$-αβ3 axis, we hypothesized that there would be an advantage in combining tetrac with bortezomib. Our results provided support for a sensitizing effect for most actions in the combined treatment. Tetrac has been shown before to increase the sensitivity of resistant cancer cells to chemotherapy (Rebbaa et al. 2008, Davis et al. 2011, 2014), via the plasma membrane multi-drug resistance (MDR) pumps.

Finally, the expression of αβ3 integrin in whole BM mononuclear cells was revealed not only on the malignant plasma clone, as previously reported (Ria et al. 2002, Tucci et al. 2009), but also on heterogeneous cell populations within the BM (monocytes, lymphocytes and promyelocytes). The expression of αβ3 on peripheral blood monocytes and granulocytes has recently been reported (Shojaei et al. 2012). To the best of our knowledge, our results are the first to indicate αβ3 abundance on monocytes, granulocyte precursors (promyelocytes) and lymphocytes in the MM BM. These results point out that the thyroid hormone-αβ3 axis may be relevant not only to the malignant clone, but also to the surrounding BM mononuclear cells. Such an assumption was recently supported by another study showing the relevance of the thyroid hormone-αβ3 axis to the crosstalk between the BM microenvironment and other malignant cells (Schmohl et al. 2015, Davis et al. 2016). This further suggests that it may be important to study primary MM cells in the context of their stromal cell milieu.

In conclusion, tetrac generated anti-proliferative and pro-apoptotic responses and increased the sensitivity to combined tetrac-bortezomib treatment in MM cells. This, together with our observation of αβ3 integrin.

Three-dimensional crystallographic modeling of the αβ3 integrin confirmed that tetrac-binding site, similar to that of the thyroid hormones, is located near the RGD-recognition site (Cody et al. 2007, Freindorf et al. 2012, Davis et al. 2016). Our results show that various tetrac-initiated effects were attenuated in the presence of RGD. Moreover, at equimolar concentrations, it appeared that tetrac was far more potent than RGD, which had no effect whatsoever, even at high micromolar concentrations. These results appear to be consistent with preclinical and clinical experience with RGD antagonists which report that high millimolar concentrations are required to produce an inhibitory effect in cancer (Weis et al. 2009).

Experiments performed on primary BM cells from MM patients often involve the enrichment of the samples with CD138+ plasma cells. However, this approach overlooks other mononuclear cells that are present in the BM. Given the recently acknowledged importance of the microenvironment in supporting MM (Rossi et al. 2013), there might be an advantage in studying the malignant clone within its natural surrounding niche. Such studies on heterogeneous cell populations in the myeloma BM have been reported (Lichtenstein et al. 1989, Liu et al. 2005, Noll et al. 2014, Chen et al. 2017, Gao et al. 2017). In this work, tetrac at low concentrations induced apoptosis in primary BM mononuclear MM cells at various disease stages. This apoptosis was evident in whole BM mononuclear cells and more profoundly in the plasma cell clone. Although no correlation between the response to tetrac and disease stage was documented, this should be examined in a larger scale study. Of note, in myeloma cell lines, the concentrations of tetrac needed to produce an apoptotic effect were generally higher. This may suggest that the mononuclear cellular milieu susceptible to tetrac indirectly assists in MM cell death.

Figure 8
Primary bone marrow cells from MM patients highly express αβ3 integrin. Flow-cytometry analysis of cells incubated with (A) αβ3 antibody and (B) CD138-APC and CD45-FITC antibodies. Malignant plasma cell clone is depicted in pink; mononuclear cells in green. (C) Analysis of expression of integrin in CD138+ (pink) vs CD138-cells (green). (D) Forward scatter (FSC) and side scatter (SSC) plot of the various cell populations: plasma cells (blue), promyelocytes (purple), monocytes (yellow) and lymphocytes (green). (E) Relative αβ3 expression in the various cell populations. Isotype control is depicted in black.
expression in primary MM and stromal cells, highlights the relevance of this integrin in this disease and suggests that pharmacologic inhibition of the thyroid hormone-integrin αvβ3 pathway may serve as a potential approach to disease management.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/ERC-17-0246.

Declaration of interest
Co-author P J D is stockholder and officer in a company developing modified forms of tetrac as anticancer agents. The other authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
K C designed, performed, analyzed and interpreted the experimental data. U A and M E assisted in the selection of patients included in the study and the collection of the BM samples. O A F designed, analyzed and interpreted the experimental data. K C, P J D, A H, M E and O A F wrote the manuscript. All authors read and approved the final manuscript.

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