Abstract. New drugs are urgently needed for improved therapy for melanoma. Materials and Methods: Ninety-one novel compounds were evaluated in two melanoma and one normal skin cell lines to identify potential lead compounds with high potency and selectivity. Mechanisms of action for the best compound were also investigated. Results: Three potent lead structures (serine amino alcohols, serine amides and thiazolidines) were identified, with thiazolidines having both excellent potency and high selectivity when compared with sorafenib, a drug used extensively in clinical trials for melanoma. Analyzing the effect of the lead compound showed that it induced DNA degradation consistent with necrotic cell death. Conclusion: The lead structure represents a novel class of compounds that can be further optimized for potential drug to treat advanced melanoma.

Melanoma is the most aggressive form of skin cancer and is the fastest growing cancer in the western world (1-4). It is also the most common cancer in young adults aged 20-30. While in situ melanoma (stage 0) can be cured surgically, melanoma metastasizing to major organs (stage IV) is virtually incurable (4). Patients with advanced melanoma have a median survival time of 7.5 months and the estimated 5-year survival rate is only 5~9% (5-8).

Currently, dacarbazine (DTIC) is the only FDA-approved drug to treat advanced melanoma, but it provides complete remission in only 2% of patients (7, 9). The FDA also approved the use of high-dose interferon alpha-2b (IFN-α2b) as adjuvant treatment of patients at high risk of recurrence of melanoma, but several recent Phase III randomized trials failed to detect a survival advantage with the addition of IFN to DTIC (10-12). Several extensive clinical trials have been conducted in recent years with a variety of cancer drugs or combination of cancer drugs, including DTIC combined with other drugs (e.g., cisplatin, vinblastine, or carmustine) (13, 14), but they all have failed to demonstrate clear effect against advanced melanoma (7, 15, 16). Therefore, DTIC still remains the gold standard for advanced melanoma despite its very limited efficacy (17, 18). With the rapidly rising incidence reported for melanoma in the world, clearly there is an urgent need to develop more effective therapeutic agents to combat advanced melanoma.

The therapeutic effect of DTIC is believed to be produced through alkylation of DNA (19). However, one of the major functions of human skin is to protect internal organs from harmful external stress, including constant UV irradiation from the sun. Since UV is particularly harmful to DNA structure, it is not surprising that skin cancer has strong resistance to existing DNA alkylating agents such as DTIC. Exploring chemotherapeutic agents with different mechanisms of action that could reduce or bypass DNA alkylation resistance should provide a better therapeutic index.

Novel classes of lipid compounds have been synthesized and have shown strong activity towards prostate cancer cells (20, 21). These classes of compounds are clearly not DNA alkylating agents, but possibly interfere with receptors in the cell membrane. To further understand the structural features and to investigate their potential for broad anticancer activity, we conducted biological evaluations using an expanded library of these compounds against melanoma in vitro. In this paper we report the structure-activity relationships of these compounds and studies of their antiproliferative activity against melanoma.
Materials and Methods

Compound library. Ninety-one compounds were selected to form a library for biological testing. The chemistry of these compounds has been reported elsewhere (20, 22). Their activity was compared with the existing drugs, Taxol and DTIC (Sigma-Aldrich, Inc., St. Louis, MO, USA). In addition, sorafenib (Bay43-9006) has recently been used extensively in clinical trials for advanced melanoma and is well tolerated in patients (23). Hence, we also synthesized sorafenib based on published procedures (24) and used its activity as a standard to assess our lead compounds.

Cell culture. SKMEL-188 cells were derived from primary tumors and cultured in Ham’s F10 medium (Gibco Invitrogen, Inc., Grand Island, NY, USA) plus 5% fetal bovine serum (FBS) (Cellgro Mediatech, Inc., Herndon, VA, USA) and 1% antibiotic/antimycotic mixture (Sigma-Aldrich Inc., St. Louis, MO, USA) (25). WM-164 cells were derived from metastatic tumors and were a gift from Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA, USA). Both WM-164 and human dermal fibroblasts (purchased from Cascade Biologics Inc., Portland, OR, USA) were cultured in DMEM (Cellgro Mediatech), supplemented with 5% FBS, 1% antibiotic/antimycotic mixture and bovine insulin (5 µg/ml; Sigma-Aldrich). Human dermal fibroblasts were used as a control to examine the effect of compounds on the stromal environment. Thus, the effect of drugs on melanoma cells versus fibroblasts would document their potential selectivity.

Sulforhodamine B assay. Cells were seeded into 96-well plates at 5000 cells/well. After 12 h, media were changed and serial dilutions of compounds were added (1 nM to 100 µM). Cells were incubated with each compound for 48 h. Fresh media containing the testing compound were changed every 24 h. Thereafter, total cell protein corresponding to cell numbers (both viable and non-viable cells) was measured using the sulforhodamine B (SRB) assay according to the manufacturer’s protocol (Sigma-Aldrich) (26, 27). Briefly, cells were fixed with trichloroacetic acid, washed, and incubated with sulforhodamine B for 30 min. After another wash, dye incorporated into the cells was solubilized and measured using a plate reader at 565 nm.

Lactate dehydrogenase release assay. SKMEL-188 cells were seeded at a density of 5000 cells per well in 96-well flat-bottom microtiter plates. Media were changed after 12 h, and the testing compound was added at serial dilutions. Media were changed with fresh compound every 24 h. After incubation for 48 h, supernatants were collected and transferred to a new plate. Lactate dehydrogenase (LDH) in supernatants was measured using Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA). Briefly, substrate mix solution (containing tetrazolium salt) was added to each well and incubated at room temperature for 30 min. At the end of incubation, stop solution was added to each well and the plate was read at 490 nm. Cytotoxicity of compounds was calculated using the following formula: % cytotoxicity = (experimental LDH release/maximum LDH release). Experimental LDH release was calculated by subtracting the absorbance corresponding to the media alone from the absorbance corresponding to control or compound-treated cells. Maximum LDH release was calculated by subtracting the absorbance corresponding to the media with added lysis solution from the absorbance corresponding to cells lysed with lysis solution.

DNA content analysis. Flow cytometry analysis was performed as described elsewhere (28). Briefly, SKMEL-188 cells were seeded in 10-cm Petri dishes at a density of 10^6 cells per dish; at 12 h the medium was changed to 5% FBS with different concentrations (0, 60 and 6 µM) of the testing compound. Cells were incubated for 48 h and media with compounds were changed every 24 h. Cells were trypsinized, washed with PBS and fixed in ice-cold 70% ethanol. Ethanol was removed by centrifugation and cells were incubated in phosphate-citrate buffer (0.2 M Na2HPO4, 4 mM citric acid, pH 7.8) for 1 h. Finally, cells were centrifuged and 1 ml of propidium iodide solution (50 µg/ml) and RNase (0.1 mg/ml) in PBS was added (29). Samples were shaken for 30 min and analyzed with a FACS Calibur cytomter (Beckton Dickinson, San Diego, CA, USA). Data were analyzed and graphs prepared using the Modfit 2.0 program (Verity Software House, Topsham, ME, USA).

Statistical analysis. Data are presented as mean±SEM (n=6-8). IC50 (i.e., concentration that inhibited cell growth by 50% of untreated control) values were obtained by nonlinear regression analysis (GraphPad Prism, San Diego, CA, USA). Statistical analyses were performed with the Student’s t-test (*p<0.05, **p<0.005).

Results

Identification of arylthiazolidine carboxylic acid amides (ATCAA) as highly active lead structures for melanoma. In the first step of lead identification, cells were exposed to each compound at 10 µM to identify those which are active for both SKMEL-188 and WM-164 cell lines. The IC50 values of the seven most active compounds with 50% or more growth inhibition at 10 µM were subsequently measured on both cancer cell lines and on the control cells (fibroblast cells). Activities of several compounds with similar structures to the active compounds were also measured in order to understand the structure-activity relationships (SAR). Sorafenib, DTIC and Taxol were assayed in the same way for comparison and served as internal quality controls in the different assay batches. The results for all three cell types are summarized in Table I.

Overall, three basic structures were found very active in inhibiting both melanoma cell lines: (A) serine amides (e.g. VG-III-15), (B) serine amino alcohols (VGII-197), and (C) arylthiazolidine-4-carboxylic acid amides (ATCAA, e.g., VG-III-39) (Figure 1). Analogs of (A) and (B) did not possess acceptable selectivity for cancer cells. Thiazolidine derivatives (C) displayed the highest potency and selectivity with IC50 values in the nanomole range for cancer cells (Table I, VG-III-39). Not surprisingly, data from this in vitro assay indicated that DTIC is inactive (IC50>100 µM) against both cancer cell lines due to the lack of bio-activation. This is consistent with the currently proposed mechanism of action for DTIC, as well as findings by Daidone and co-workers (30). Clearly, in vivo assay is needed to assess the true activity of DTIC and similar structures. While Taxol is outstanding in inhibiting cancer cell growth, it is not effective in killing melanoma cells (cytostatic). In contrast, all the lead compounds from these preliminary
studies killed the cells completely at higher concentrations. The mechanisms of action of our compounds are clearly different from that of DTIC and Taxol.

Sorafenib effectively killed all the melanoma cells at high concentrations, similar to that of our lead compounds. When measured by IC₅₀ values, VG-III-39 is about 10 times

Table I. IC₅₀ measurement of the lead compounds and their comparison with that of Sorafenib, DTIC and Taxol (ND: data not available). *Taxol only killed about half of the cells while all other compounds except DTIC kill all the cancer cells at high concentration.
as potent as sorafenib. However, its selectivity, defined as the ratio of IC\textsubscript{50} in fibroblast cells over IC\textsubscript{50} in melanoma cells, is moderate when compared with that of sorafenib (10 vs. larger than 25). This is consistent with the clinical finding that sorafenib has very low toxicity and is well tolerated by patients (23). The potency and selectivity of sorafenib provide an excellent standard to assess the activities of our compounds and represent the future goal for optimization of our lead structures.

**VG-III-39 induces cancer cell death by necrosis.** To examine more closely the cell death induced by our compounds, we measured lactate dehydrogenase (LDH) release by colorimetric assay and analyzed DNA content by flow cytometry.

LDH is a stable cytosolic enzyme that is released upon cell lysis. Treatment of cells with VG-III-39 resulted in a dramatic dose-dependent increase of LDH released to the cellular environment (Figure 2A).

Analysis of the DNA content of control cells (Figure 2B) showed peaks corresponding to the cells in G1/0-cell phase (62%), S-phase (32%) and G2/M-phase (6%), a typical distribution of cells in culture (29). Treatment of cells with VG-III-39 at 6 μM and 60 μM led to the disappearance of peaks corresponding to viable, proliferating cells and the appearance of single peaks corresponding to debris/degraded DNA. This appearance is typical for cells undergoing necrosis as opposed to cells undergoing apoptosis (29, 31). Apoptotic cell death is usually characterized by an apparent sub-G1 peak between cellular debris and the G1/0 peak (31). To further confirm that VG-III-39 does not induce significant apoptosis, we analyzed its effect on the activation of effector caspase-3. Indeed, VG-III-39 did not increase its activity in the cells (results not shown).

**Discussion**

Due to the rapidly rising incidence of melanoma and the limited efficacy of existing drugs, new drugs with novel mechanisms of action are urgently needed. One promising drug development strategy for cancer involves identifying and testing agents that interfere with growth factors and other molecules involved in cancer cell signaling pathways. For example, protein kinase B-Raf mutation has recently been identified as a viable drug target due to its high occurrence in the majority of melanoma tissues (32). A Phase II study of Bay 43-9006 (sorafenib), a potent RAF kinase/VEGF kinase inhibitor, demonstrated promising effects against melanoma (33, 34). Developing chemotherapeutic agents against novel targets or combination of targets could prove to be very fruitful for combating advanced melanoma.

In this report we examined novel classes of compounds and identified three lead structures that are highly potent for melanoma. These compounds clearly have mechanisms of actions that are different from existing drugs for melanoma. When comparing the activity with that of sorafenib, our lead compound (VG-III-39) is about 10 times as potent, although its selectivity could be improved further. Several important SARs were revealed in examining the 91-member library. Firstly, amides were less active than the corresponding alcohols (Figure 1 A and B respectively). Heteroatoms on the side chain substantially increased the potency (VG-III-15), but unsaturation on the side chain had the opposite effect (VG-II-189). Secondly, an alkyl amine (VG-II-197) was about equipotent to the un-substituted amine (VG-II-201), suggesting that cyclization of the amine and alcohol may be possible. However, none of the open chain compounds displayed acceptable selectivity against cancer cells. Thirdly, the presence of the aromatic ring in the

![Figure 1. Identified lead structures.](image)

**Figure 2. Compound VG-III-39 caused necrotic cell death.** SKMEL-188 melanoma cells were incubated with VG-III-39 for 48 h. A) LDH released from the cells after treatment was then analyzed. Data is presented as mean±SEM (n=8), *p<0.05, **p<0.005. B) DNA content was analyzed using flow cytometry. Results are representative of two separate experiments.
heterocyclic compounds improves selectivity and cytotoxicity of structure C (Figure 1) (VG-III-215 vs. VG-III-149). Fourthly, the potency of the compounds depended strongly on the chirality at the C-4 position (VG-III-41 vs. VG-III-149), the substitution on the aromatic ring (VG-III-149 vs. VG-III-265), and the chain length (VG-III-39 vs. VG-III-149). With a similar C18 chain, the S-isomer at the C-4 position (VG-III-41) showed higher potency than the R-isomer (VG-III-149). More electron donating substitutions on the aromatic ring progressively increased the potency of analogs of structure C (VG-III-149 vs. VG-III-83, VG-III-267, and VG-III-265). N-substitution with the C14 chain was much more potent and selective against cancer cells than that of the C18 chain compound (VG-III-39 vs. VG-III-149). Clearly, further structural optimization is warranted and will likely improve both potency and selectivity for this class of compounds against melanoma.

It is interesting to compare the activity in melanoma with that in prostate cancer for these compounds. In general, their activities are consistent in these two types of cancer cells, demonstrating potentially broad anticancer properties for ATCAA analogs. Compounds that are active or inactive in prostate cancer also displayed similar behavior in melanoma, but there are a few exceptions. For example, although VG-III-149 (C18 chain length) showed excellent activity (IC50<3 µM) against prostate cancer cells (20), it was not active in either melanoma cell line (IC50>40 µM). In addition, for compounds that are active in both cancer types, their relative potency and selectivity are not the same. For example, in prostate cancer studies, a compound with a C14 side chain was found to be slightly less active than that with a C18 chain (20). Substitutions on the aromatic ring with mono-, di- or trimethoxyl groups were found to be favorable in prostate cancer cell lines. Both the potency and selectivity increased as more methoxyl substitutions were added to the aromatic ring (20). In melanoma, however, methoxyl substitutions did not have significant selectivity benefits when compared with nonsubstituted analogs (e.g. VG-III-83 vs. VG-III-265). A C14 chain displayed much better activity and selectivity than C18 chain compounds (e.g. VG-III-39 vs. VG-III-265). Interestingly, Taxol behaves very differently in prostate cancer cells and melanoma cells. While Taxol killed all the prostate cancer cells, it only killed about half of the melanoma cells when compared with controls based on our in vitro assay (unpublished data). This may suggest different oncogenic properties between these two types of cancer.

Although the mechanisms of action for these compounds are not entirely clear at this stage, our LDH release studies showed that these compound induced cell death through necrosis instead of apoptosis (Figure 2). Further studies are in progress in order to fully elucidate the target for this class of compounds in order to potentially guide the lead optimization process.

Conclusion

ATCAA analogs represent a novel class of potent and selective cytotoxic agents for melanoma. Initial analysis indicated that the antineoplastic activity is attributed to their ability to induce necrosis in melanoma cells. These SAR studies revealed that the potency of the compounds depends strongly on the C-4 chirality, chain length and substitutions on the phenyl ring. Our current lead compound is highly potent and moderately selective when compared with sorafenib, a drug used extensively in clinical trials for melanoma. Clearly these compounds have great potential if further optimized. In light of these results, we are currently synthesizing more analogs of structure C to optimize both the potency and selectivity against melanoma cells. We seek to understand the optimal chain length and chirality at the C-4 position. The most potent analogs of structure C as pure optical isomers will be prepared for future in vivo studies in animal models.

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References


