SPECTRAL ANALYSIS OF HUMAN NOREPINEPHRINE TRANSPORTER HOMING PEPTIDES

YAZAN HADDAD[1,2], VEDRAN MILOSAVLJEVIC[1,2], LUKAS NEJDL[1,2], LUKAS RICHTERA[1,2], ZBYNEK HEGER[1,2], VOJTECH ADAM[1,2]

[1]Department of Chemistry and Biochemistry
Mendel University in Brno
Zemedelska 1, 613 00 Brno
[2]Central European Institute of Technology
Brno University of Technology
Purkynova 123, 612 00 Brno
CZECH REPUBLIC

yazanhaddad@hotmail.com

Abstract: UV spectrometry is very simple and cheap method for quantitative and qualitative analysis of compounds. Furthermore, it provides intricate information about the bonded π-electron transitions and also non-bonded n-electron transitions. The aim of this work was to identify electron transition bands in two homing peptides of the human norepinephrine transporter (hNET); namely: GASNGINAYL (978 Da) and SLWERLAYGI (1206 Da). Electron transition bands directly indicate structural conformations, particularly the ones associated with double bonds, i.e. conjugated π-bonds of aromatic and peptide bonds. The results show unusual spikes in absorbance in the far UV at low temperature for GASNGINAYL and even more at other temperatures for SLWERLAYGI peptide. The latter supports the hypothesis of a stacking between tyrosine and tryptophan resulting in helix structure. Infrared spectrometry also showed abundant helix structure in SLWERLAYGI but less abundant in GASNGINAYL peptide. Based on π-stacking, an UV spectrometry method can be developed to monitor the helicity of some peptides, such as SLWERLAYGI.

Key Words: UV spectrometry, peptide, tyrosine, tryptophan, targeted therapy

INTRODUCTION

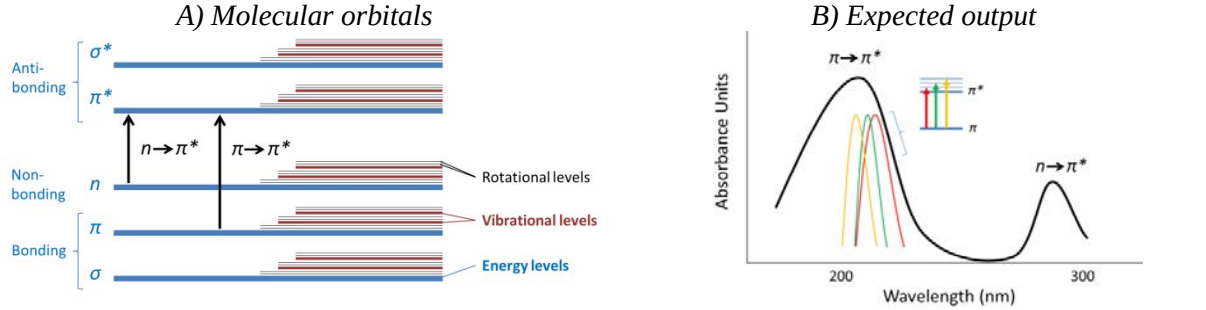
The spectral analysis of amino acids and polypeptides in the UV region was intensively studied more than fifty years ago (Ham and Platt 1952, Wetlaufer 1963, Nielsen and Schellman 1967). UV-Vis spectrometers are used to analyse absorbance of photons that excite electrons to jump from the non-bonding and bonding orbitals to anti-bonding orbitals (Figure 1). Electrons of the π-bond (in double bonds) and free electrons (in the n-orbitals) exhibit unique and quantitative band fingerprints for each molecular structure and in the presence of different solvents, pH and temperature.

The peptide group –CONH– contains four π-electrons and free electrons on the oxygen atom. The energies of the two π-orbitals occupied by electron pairs are -15.04 and -12.68 electron volts (eV). The nonbonding n-orbital at -12.63 eV and the free anti-bonding orbital at +1.24 eV (Simon 1976). Aromatic amino acids such as tryptophan and tyrosine are rich in conjugated π-electrons, and thus have direct influence on absorbance in the UV-Vis region.

Kuipers and Gruppen studied the absorbance of peptide bond at 214 nm (Kuipers and Gruppen 2007). Their findings showed a molar extinction coefficient (ε) of 923 1/M.cm for the peptide bond. This parameter, which is specific for each wavelength, describes how strongly one molar concentration of peptide bond can absorb photons in 1 cm light-path. At 214 nm, tryptophan had highest absorptivity (ε = 29050 1/M.cm). Tyrosine, phenylalanine and histidine reported similar absorptivity (ε = 5375, 5200, and 5125 1/M.cm, respectively) at that wavelength, whereas methionine had similar ε to that of the peptide bond (ε = 980 1/M.cm). Proline had three times higher ε when it is at the *N*-terminus (ε = 2675 1/M.cm) but yet negligible ε inside the peptide chain. Several studies also investigated the peptide bond absorptivity at 205 nm (Scopes 1974, Anthis and Clore 2013). Anthis

and Clore showed ε = 2780 1/M.cm for the peptide bond (Anthis and Clore 2013). They also reported ε for tryptophan (ε = 20400 1/M.cm), phenylalanine (ε = 8600 1/M.cm), tyrosine (ε = 6080 1/M.cm), histidine (ε = 5200 1/M.cm) and other amino acids.

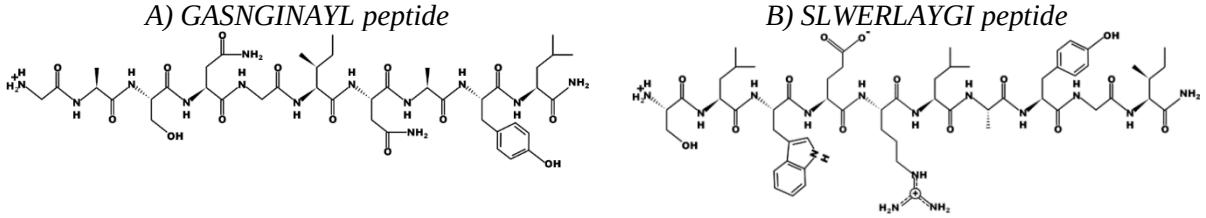
Figure 1 Molecular orbitals and electron transitions detectable via UV-Vis spectrometry



Calculation of sequence-specific ε values for a wide range of wavelengths in the UV spectra can give accurate estimations on the contribution of residues and peptide bonds to spectral bands. While this is applicable for quantitation of large proteins, UV spectrometry can also be used to give insight on structural changes in relatively small peptides of known sequence.

In this study, two homing peptides (shown in Figure 2) are analysed by spectrometry to shed the light on their structural properties. Our objective is to extract structural information from UV spectrum that can help in development of these peptides. Both peptides were derived from α-helix regions in hNET and were shown to have α-helix secondary structure *in silico* (Haddad et al. 2016). hNET protein is one of the most promising therapeutic targets in Neuroblastoma (Matthay et al. 2012). Development of homing peptides for hNET is a new strategy that can replace radio-therapeutic targeting of hNET, and ease the suffering of patients.

Figure 2 Norepinephrine transporter homing peptides structures at pH = 7



MATERIAL AND METHODS

Peptide Synthesis

Peptides were synthesized on Liberty Blue synthesizer (CEM, NC, USA) and stock solutions of 1 mM were prepared in ACS water.

UV-Vis Spectrometry

Specord S600 spectrometer (Analytik Jena AG, Germany) was used for analysis. Temperature of the spectrometer chamber was controlled manually using JUMO dTRON 308 regulator (Analytik Jena AG, Germany). Quartz cuvettes of 1 cm path were used (Hellma, UK). Samples were equilibrated at each temperature for three minutes prior to each reading. ACS water was used as reference. Spectra, measured at 0.5 nm resolutions, were normalized at 280 nm point for peptides at each temperature separately. Peaks were identified manually as highest point(s) with one or two shoulders.

Attenuated Total Reflectance Fourier Transform-Infrared Spectroscopy (ATR-FT-IR)

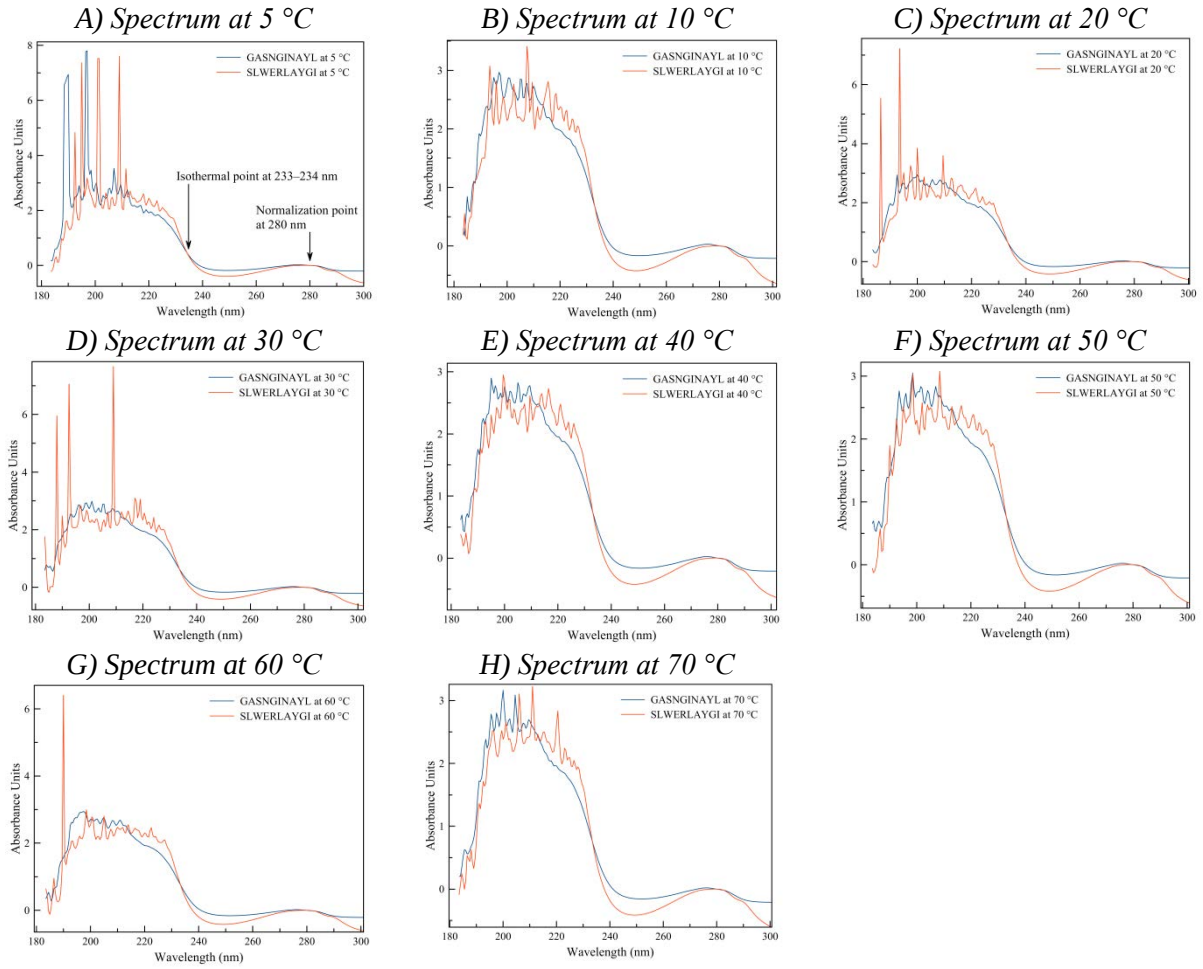
FT-IR spectra were collected using a Nicolet iS10 FT-IR spectrometer with attenuated total reflection (ATR) attachment (Thermo Fisher Scientific, USA), equipped with diamond crystal. Spectra were recorded at 25 °C from 4000 to 650 1/cm at a resolution of 2 1/cm. Each spectrum was acquired by merging 128 interferograms. Peptide samples were directly analysed in lyophilized form.

RESULTS AND DISCUSSION

The double bonds, particularly conjugated double bonds in aromatic structures and peptide bonds, are rich of π-electrons that can be excited in the near and far UV spectrum. The near and far UV spectrum can be used to identify overlapping yet sometimes distinguishable π→π*, n→π* and other electronic transitions. Indeed, the overlapping peaks (as predicted in Figure 1B) around the 220 nm can be attributed to n→π* transition that overlaps the π→π* region in addition to other hidden electronic transitions (Wetlaufer 1963). The shoulder of this peak is slightly red shifted in SLWERLAYGI peptide due to larger number of double bonds in its structure when compared to GASNGINAYL peptide (Figure 3). Researchers use different spectrometric methods to resolve the type of electron transition, such as UV-Vis spectrometry analysis in presence of different solvents, pH titration and substitution with different chemical groups.

The UV spectral peaks for GASNGINAYL peptide at different temperatures are summarized in Table 1. There are several "saturated" spikes in the spectrum particularly at low temperature (Figure 3A). The spikes correspond to 6.61–6.53 eV and 6.33–6.29 eV energy bands at 5 °C (Table 1). We believe these spikes belong to the aggregation and stacking of the tyrosine aromatic rings in the peptides. The fact that these spikes are in the far UV region suggests that they are a result of π→π*, however further confirmation is required.

Figure 3 UV Spectra of the hNET homing peptides



The UV spectral peaks for SLWERLAYGI peptide are summarized in Table 2 at different temperatures. This peptide contains an additional aromatic residue (i.e. tryptophan). As expected, more saturated spikes were identified in low temperature (Figure 3A). The spikes correspond to 6.44–6.42 eV, 6.36 eV, 6.18–6.15 eV and 5.93–5.88 eV energy bands at 5 °C (Table 1). Surprisingly, the spikes do not disappear at higher temperatures (Figure 3C, 3D and 3G). Energy bands of these spikes at 20 °C are 6.68–6.65 eV and 6.42–6.41 eV. And at 30 °C the energy bands

for spikes are 6.61–6.56 eV, 6.44–6.42 eV, and 5.93–5.88 eV. Another spike appears again at 60 °C in the energy band 6.56–6.47 eV. These results demonstrate that the tyrosine and tryptophan stacking is rather a phenomenon inside the peptide and not between peptide aggregates. Unfortunately, we have no data on the spectral behaviour of individual tyrosine or particularly tryptophan under different temperatures. Indeed, tyrosine lost these spikes at higher temperatures; however, it was not possible to know if tryptophan alone exhibits more excited electrons at higher temperature. Esfandiary et al. reported gradient shift in the peaks of aromatic amino acids in the 250–300 nm region that correlate with change in temperature, however such shifts did not exceed 1nm in 10–60 °C range (Esfandiary et al. 2009).

Table 1 Spectrum peak wavelengths (nm) for GASNGINAYL peptide at different temperatures

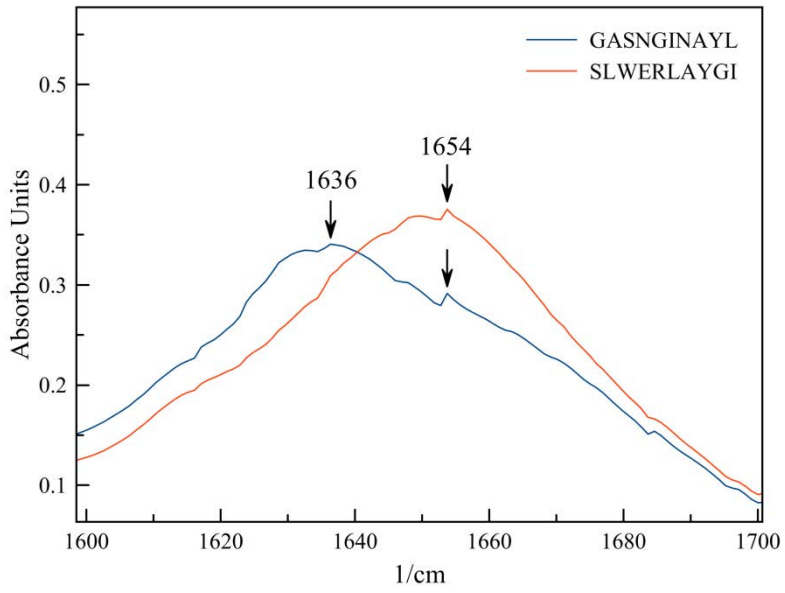
Energy (eV)	70 °C	60 °C	50 °C	40 °C	30 °C	20 °C	10 °C	5 °C
6.76–6.72		184.5	184	184	184	183.5	183.5	
6.70–6.65	185.5–186	186.5	186	186	185.5		185	185
6.61–6.53					188.5		187.5	188.5–190
6.56–6.47	191	190.5	189–189.5	190	191–191.5	190	189.5	
6.46–6.44				192		192.5		
6.46–6.41	193.5	193	193.5	193.5	193–193.5		192–192.5	192.5–193
6.39–6.33	195.5	194.5	195.5–196	195		194.5–195	195	194
6.33–6.29				196.5	196–196.5			196.5–197
6.33–6.23	197.5	196–197.5	198.5	198.5	198.5–199	198.5–199	197–197.5	198.5
6.20–6.18	200	200–200.5	200.5	200		200		200.5
6.18–6.17					201		200.5–201	
6.15–6.12	202.5	202–202.5	201.5–202	202–202.5		201.5–202	202	
6.11–6.09					203.5	203.5		203
6.08–6.03	204.5	204.5–205	204–204.5	205–205.5	205–205.5	205.5	205–205.5	205–205.5
5.99–5.98			207	207–207.5	207		207–207.5	207
5.98–5.93	207.5–208	208–208.5	208.5		208.5–209	208–208.5		
5.93–5.88	209.5–210	210.5–211	211	209–209.5	210.5	210–210.5	210	209.5
5.85–5.81		213	213	212.5–213	212.5–213	212	213–213.5	212
5.78								214.5
5.77–5.69	218			216–216.5	216.5	216.5–217	215–216	216.5–217
5.67–5.66						218.5–219	218.5–219	219
5.65–5.61	219.5–220	220		219.5–220	220	220.5	220.5–221	
5.60–5.55	223		221.5	221.5–222.5	222.5	223–223.5	223.5	223–223.5
5.51–5.50							225–225.5	

ATR-FTIR is a useful method for analysing vibrational frequency of different amide modes. Amide I is one of the most widely used mode in protein structural studies (Adochitei and Drochioiu 2011). Based on infrared spectra of many proteins, bands in the range 1650–1658 1/cm were found to be correlated with α-helix secondary structures while bands in the range 1620–1640 1/cm correlated with β-sheet secondary structures (Haris and Chapman 1992). Another study indicated that the β-sheet range was between 1621 and 1640 1/cm followed by random coil region at 1641–1647 1/cm, helix at 1648 1/cm, turns and bends at 1658–1696 1/cm which is also separated by a β-sheet gap at 1671–1679 1/cm (Wilson et al. 2000). In this study, the amide I band showed a predominant peak in the α-helix range for SLWERLAYGI peptide as expected (Figure 4). Surprisingly, GASNGINAYL peptide showed major amide I peak at the β-sheet range, in addition to a minor spike in the α-helix range. It is unclear if this was a direct result of *C*-terminal amidation during peptide synthesis. The α-helix conformation of SLWERLAYGI peptide allows for stacking of tyrosine and tryptophan which can explain the unusual $\pi \rightarrow \pi^*$ transitions peaks in UV spectra (Figure 3A, 3C, 3D, and 3G). Similar saturated peaks for UV spectra of GASNGINAYL peptide occurs only at 5 °C, possibly due to aggregation. The role of π-stacking is well documented in protein structures. The most common form is an off-centred parallel stacking of the aromatic rings, followed by less common T-shaped stacking (McGaughey et al. 1998).

Table 2 Spectrum peak wavelengths (nm) for SLWERLAYGI peptide at different temperatures

Energy (eV)	70 °C	60 °C	50 °C	40 °C	30 °C	20 °C	10 °C	5 °C
6.76–6.72	184.5	183.5		183.5	183.5		184	
6.68–6.65	186.5	186.5	186.5	185.5		186.5	186–186.5	185.5
6.61–6.56	188		189	188.5–189	188		188.5	187.5
6.56–6.47	191	190	190	191	190		190.5–191.5	189–189.5
6.44–6.42	192.5		192.5	192.5–193	192.5			192.5
6.42–6.41		193–193.5				193.5	193.5	
6.36			195	195		195		195
6.33–6.28	196–196.5	196		197	196.5–197	197.5	196	197
6.25		198.5	198.5				198.5	
6.23–6.20	199.5		200	199.5	199	200		
6.18–6.15	201	200.5–201			201			201–201.5
6.15–6.11	202.5	202.5–203	202	201.5–202	202.5–203	202.5	202.5	
6.09–6.08			204	203.5				204
6.06–6.00	206	205	205		205.5	204.5		206–206.5
6.00–5.98		207.5		206.5–207	207			
5.98–5.95			208.5	207.5			207.5	
5.93–5.88	211	210	210.5	209.5	209	209.5	209.5	209
5.86–5.82	213	212		212	212	212.5–213	211.5	211.5
5.82–5.78		213.5–214	213	213.5–214	214.5			213
5.78–5.70	215–215.5	216.5	216.5	216.5	217–217.5	216	215.5	214.5
5.70–5.66			217.5–218		219		218–218.5	218
5.64–5.60	220.5	220–220.5	220.5–221	221	220.5	221.5	220–220.5	220
5.58–5.55	223–223.5	222.5	223–223.5		222–222.5		222–222.5	223
5.54–5.51	224.5–225	224		224	224	224	224.5–225	225
5.50–5.46	226.5		225.5–226	226	226	226.5	226.5–227	
5.46–5.41	228	227–227.5	228		227.5	228		228–229

Figure 4 ATR-FTIR Spectrum of amide I band for the hNET homing peptides



Circular dichroism (CD) can be used to directly separate the bands in the UV spectra via polarization of light beams. CD spectrometry is the key method for determination of secondary structure in peptides and proteins. More insights on the structure of peptides can be gained by application of molecular dynamic computations as well as wet lab techniques such as Raman spectrometry and nuclear magnetic resonance (NMR). Detailed analysis of the UV spectrum can be done using mathematic derivatives to accurately point the peaks and curvature of spectrum curve (Kus et al. 1996) or the use of deconvolution to resolve hidden peaks (Antonov and Stoyanov 1993).

CONCLUSION

UV spectral analysis provides insights on peptide conformations and structure based on knowledge of electron transitions between molecular orbitals. Using peptide UV spectra at different temperatures, we identified over 25 bands that correspond to peaks in the 185–230 nm range. Also, we highlight some bands that might correspond to π-stacking of tyrosine and/or tryptophan and can be used to monitor the helicity of peptides such as SLWERLAYGI.

ACKNOWLEDGEMENTS

The research was financially supported by the Czech Science Foundation (project GA CR 17-12816S).

REFERENCES

Adochitei, A. Drochioiu, G. 2011. Rapid characterization of peptide secondary structure by FT-IR spectroscopy. *Revue Roumaine de Chimie,* 56(8): 783–791.

Anthis, N.J. Clore, G.M. 2013. Sequence-specific determination of protein and peptide concentrations by absorbance at 205 nm. *Protein Science,* 22(6): 851–858.

Antonov, L. Stoyanov, S. 1993. Analysis of the overlapping bands in UV-vis absorption spectroscopy. *Applied spectroscopy,* 47(7): 1030–1035.

Esfandiary, R., Hunjan, J.S., Lushington, G.H., et al. 2009. Temperature dependent 2nd derivative absorbance spectroscopy of aromatic amino acids as a probe of protein dynamics. *Protein Science,* 18(12): 2603–2614.

Haddad, Y., Heger, Z. Adam, V. 2016. Neuroblastoma Homing Peptide Screening using Unrefined Homology Structure of Norepinephrine Transporter. In *Proceedings of International PhD Students Conference Mendelnet 2016.* Brno, Czech Republic, Brno: Mendel University in Brno, Faculty of AgriSciences, pp. 983–988.

Ham, J.S. Platt, J. 1952. Far UV spectra of peptides. *The Journal of Chemical Physics,* 20(2): 335–336.

Haris, P.I. Chapman, D. 1992. Does Fourier-transform infrared spectroscopy provide useful information on protein structures? *Trends in Biochemical Sciences,* 17(9): 328–333.

Kuipers, B.J.H. Gruppen, H. 2007. Prediction of molar extinction coefficients of proteins and peptides using UV absorption of the constituent amino acids at 214 nm to enable quantitative reverse phase high-performance liquid chromatography-mass spectrometry analysis. *Journal of Agricultural and Food Chemistry,* 55(14): 5445–5451.

Kus, S., Marczenko, Z. Obarski, N. 1996. Derivative UV–VIS spectrophotometry in analytical chemistry. *Analytical Chemistry,* 41(6): 899–927.

Matthay, K.K., George, R.E. Yu, A.L. 2012. Promising Therapeutic Targets in Neuroblastoma. *Clinical Cancer Research,* 18(10): 2740–2753.

McGaughey, G.B., Gagné, M. Rappé, A.K. 1998. π-Stacking interactions alive and well in proteins. *Journal of Biological Chemistry,* 273(25): 15458–15463.

Nielsen, E.B. Schellman, J.A. 1967. The absorption spectra of simple amides and peptides. *The Journal of Physical Chemistry,* 71(7): 2297–2304.

Scopes, R. 1974. Measurement of protein by spectrophotometry at 205 nm. *Analytical Biochemistry,* 59(1): 277–282.

Wetlaufer, D. 1963. Ultraviolet spectra of proteins and amino acids. *Advances in Protein Chemistry,* 17: 303–390.

Wilson, D., Valluzzi, R. Kaplan, D. 2000. Conformational transitions in model silk peptides. *Biophysical Journal,* 78(5): 2690–2701.