A common pharmacophore for epothilone and taxanes: Molecular basis for drug resistance conferred by tubulin mutations in human cancer cells

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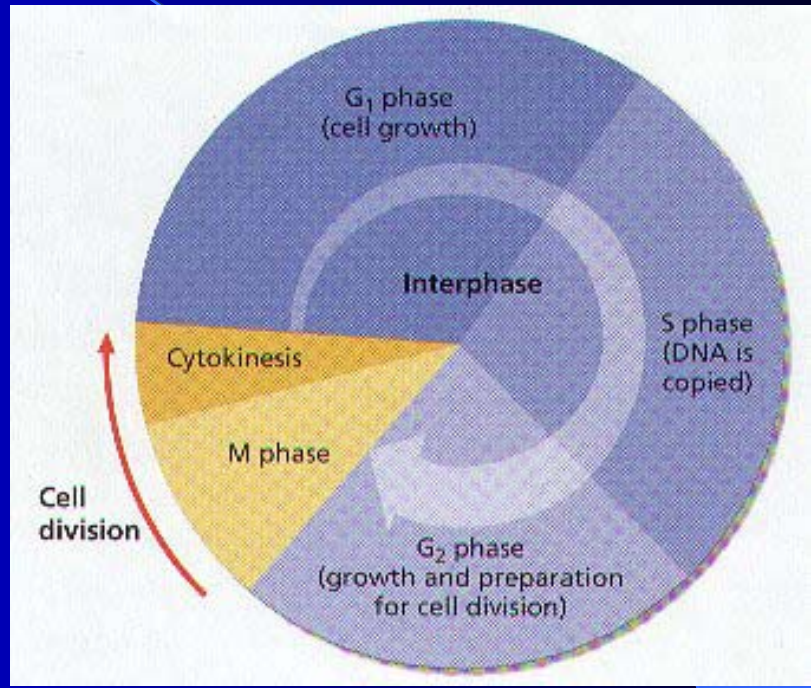
OVERVIEW

- Review
- Stabilization of Microtubules
- Goals of Experiment
- Procedures/Results
- Sarcodictyins
- Conclusion
- My Research Project
- References/Acknowledgements
- Supplemental Slides

The Cell Cycle

- M phase
  - Cell division; Each cell gets 1 copy of the genome
- G1 phase
  - Cell growth; Preparation for DNA replication
- S phase
  - DNA synthesis (replication)
- G2 phase
  - Preparation for M phase

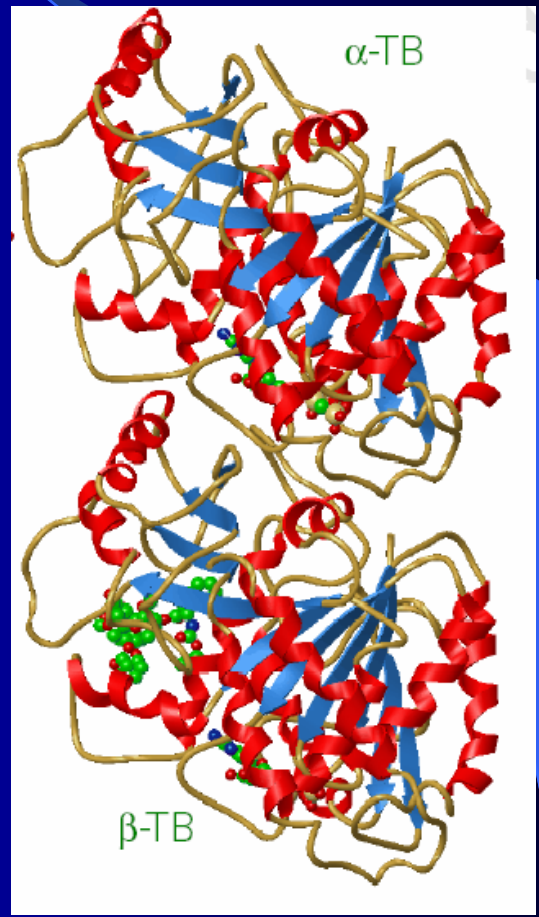


Stabilization of microtubules leads to mitotic arrest

What Are Microtubules/Tubulin?

- **Microtubules (MTs)**: cytoskeletal elements located in a cell that are essential for intracellular transport and cell division in eukaryotes; Form two functions…
  - Serve as mechanical reinforcing rods for the cytoskeleton
  - Serve as the tracks for two classes of motor proteins (kinesin & dynein)

- **Tubulin**: αβ dimer that is the structural subunit of microtubules
  - The α- and β-tubulins share 40% amino-acid sequence identity; Both exist in several isotopes; Both undergo post-translational modifications
  - Each monomer is formed by a core of two β-sheets surrounded by α-helices



Natural Product Classes That Stabilize Microtubules

- Taxane class
  - Paclitaxel (PTX)
  - Docetaxel
- Nontaxane class
  - Epothilones A and B : soil bacteria-derived
  - Discodermolide : marine sponge-derived
  - Sarcodictyin/Eleutherobins : coral-derived

Why Research Epothilones?

- Naturally occurring antimitotic drugs
- Binding sites on tubulin are unknown
- Water soluble
- Produced in large quantities through bacterial fermentation
- Retain activity against multidrug-resistant (MDR) cell lines and tumors

Tubulin Binding

- PTX binding
  - Identified by electron crystallography and photoaffinity labeling
  - Results: important areas are β1-31 and β217-233
- Epothilones
  - Binding sites not available
  - Epothilone-resistant human cancer cell lines with acquired β-tubulin mutations
    - Residues involved β274 and β282

In This Experiment…

- Form a single pharmacophore unifying taxane, epothilone, and sarcodictyin chemistries by…
  - Molecular modeling
  - Mutation data
  - Activity profile of several MT-stabilizing agents against cell lines with mutant tubulins
- Goals
  - Have a pharmacophore which explains the stabilizers' structure-activity relationship profile against both the parental and mutant tubulins
  - Provides a framework to study drug-tubulin interactions that should assist in the rational design of agents targeting tubulin

The Procedure

1. Cell Culture and Cytotoxicity Assay
2. Tubulin Polymerization Assay
3. PCR and Sequencing of β-Tubulin
4. Molecular Modeling Methods

1. Cell Culture and Cytotoxicity Assay

- Epo^R cell lines isolated after exposure of human ovarian carcinoma cell lines to lethal concentrations of Epo A or B
- After initial expansion, concentration of epothilones in culture medium gradually increased
- Cells were maintained in the drug
- Cytotoxicity assays performed in 96-well plates by seeding 500 cells per wall and incubating with cytotoxic agents for 4 days

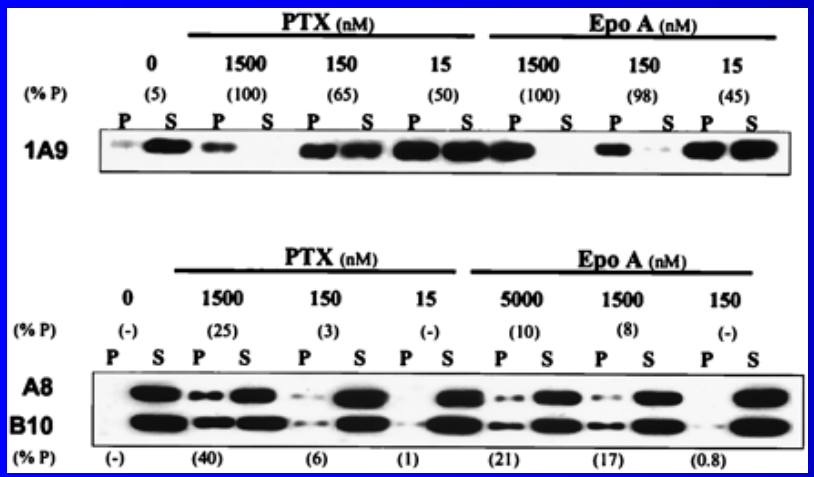
Results: Cytotoxicity Profile of 1A9/Epo^{R} Cells to Drugs Acting on Microtubules (nM)

Drug	**1A9**	**1A9/A8 (β274)**	**1A9/B10 (β282)**	Cross Resistance
PTX	2±0.23	19.5±1.14	13±0.58	10/6.5
Docetaxel	1±0.15	7±0.45	5±0.25	7/5
Epo A	3.2±0.42	127±14.7	182±6.25	40/60
Epo B	0.22±0.03	5.4±0.64	5.2±0.35	25/25
B-pyridine	0.1±0.015	3±0.25	6±0.2	30/60

2. Tubulin Polymerization Assay

- Quantitation of degree of *in vivo* tubulin polymerization due to stabilizing agents was performed
- Cells plated in 24-well plates and exposed to increasing concentrations of PTX or epothilones for 5 hours
- Cells lysed with hypotonic buffer
- Centrifugation separated cytoskeletal and cytosolic fractions containing polymerized/soluble tubulin
- Fractions then resolved by electrophoresis and immunoblotted with an antibody against β-tubulin

Results: Impaired Tubulin Polymerization



Results from a representative experiment of four independent observations

- Percent of polymerized tubulin (%P) was determined by dividing densitometric (optical density) value of polymerized tubulin by the total tubulin content (S+P)

Results: Impaired Tubulin Polymerization

- Both resistant clones demonstrated impaired *in vivo* epothilone-driven tubulin polymerization compared to parent cells
  - **Parent cells**
    - 150 nM Epo A polymerized 98% of tubulin
  - **Epo^{R} clones**
    - 5,000 nM Epo A unable to induce substantial tubulin polymerization
- PTX-driven polymerization was impaired also
  - **Parent cells**
    - 1500 nM PTX polymerized 100% of tubulin
  - **Epo^{R} clones**
    - 1500 nM PTX polymerized 25-40% of tubulin
- Therefore, cells have a higher resistance to Epo A than to PTX
- Suggests resistant phenotype resulted from impaired Epo interaction with tubulin

3. PCR and Sequencing of β-Tubulin

- PCR (polymerase chain reaction) amplification and sequencing of predominant β-tubulin isotope was performed with overlapping sets of primers
  - PCR, found to be both sensitive and quantitative, was utilized to evaluate differences in mRNA expression
  - Accurate quantitation required a demonstration of an exponential range which varied among samples.
  - The exponential range was determined by carrying out the PCR for a fixed number of cycles on serial dilutions of the RNA reverse transcription product, or by performing the reaction with a varying number of cycles on a fixed quantity of cDNA.

Results: Mutations in β-tubulin

Clone	Residue	Mutation
PTX10	270	Phe→Val
PTX22	364	Ala→Thr
1A9/A8	274	Thr→Ile
1A9/B10	282	Arg→Gln

Mutations cluster in space together → Spatial clustering on region defined as taxane binding site plus cross-resistance patterns reinforces that epothilones share a common binding site with taxanes!

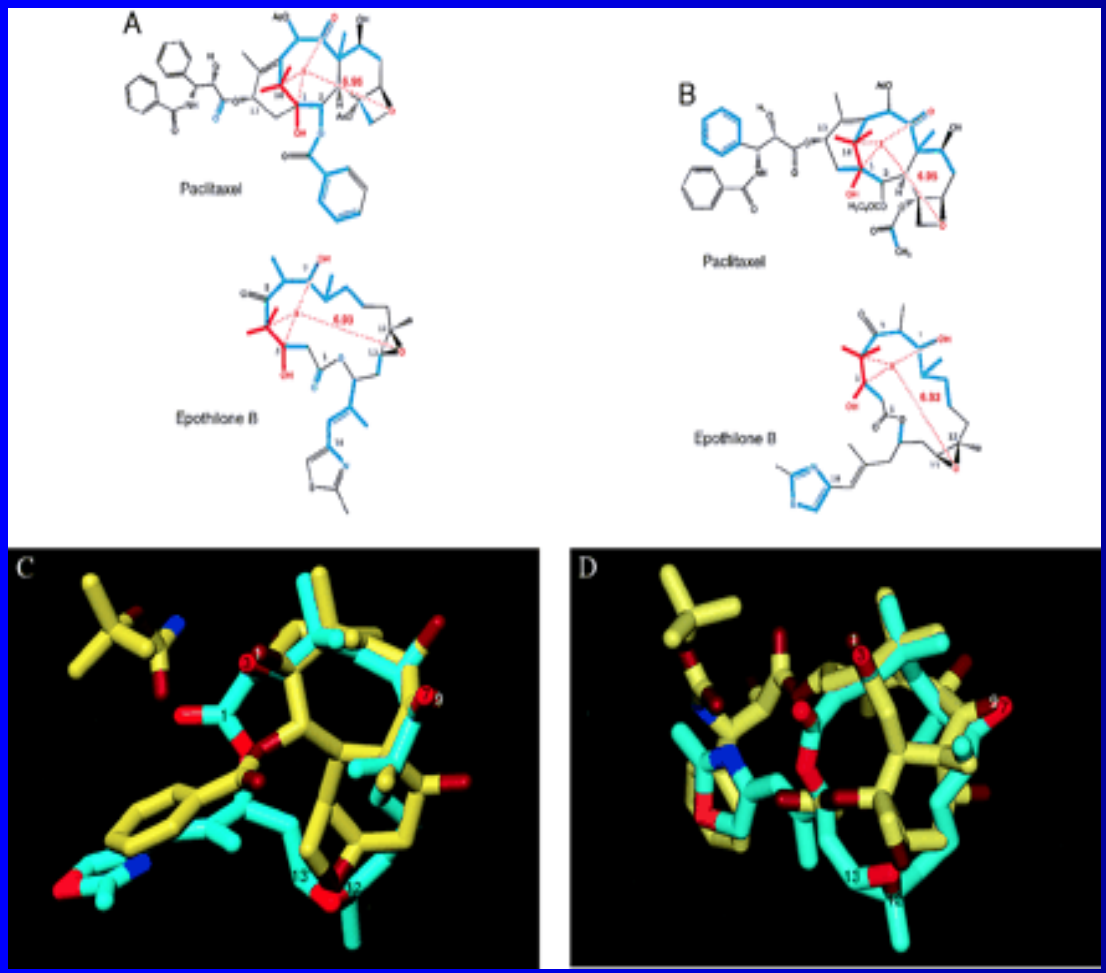
4. Molecular Modeling Methods

- Initial model of Epo B was optimized (made as functional as possible)
- Molecular mechanics potentials were selected to optimize close as possible to the crystal structure
- High temperature molecular dynamics generated low-energy Epo conformations by overcoming the potential energy barrier (hill climbing)
- Tracking distances between the epoxide O and other atoms identified common pharmacophore with taxanes and analyzed conformers of Epo
- Template forcing modeled Epo/docetaxel overlap model
- Tethered minimizations energy refined deposited tubulin structure
- Epo was docked into former binding site while docetaxel was completely ligated by water and optimized

Results: Molecular Modeling

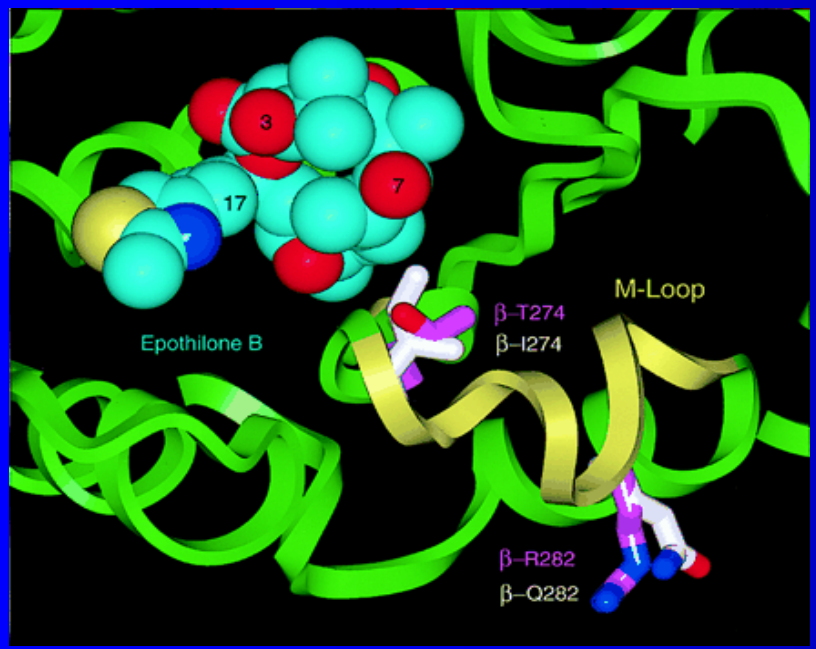
- Resulted in 100 low-energy conformationally distinct Epo structures
- On the Way to a Common Pharmacophore, but…
  - The common pharmacophmore between taxanes and epothilone should…
  - Be observable within a set of low-energy conformational isomers of epothilones
  - Consistently overlap in conformational space with the taxanes
  - Sterically fit into the taxane-tubulin binding site model in a manner consistent with relative mutation data
- These 3 criteria reduced possible conformational Epo isomers to 2!

Results: 2D Pharmacophore Overlap of Epo B and PTX



- The centroid of 3-OH, 7-OH, and 4-gem-dimethyl groups of Epo fell at a mean distance of 6.93 Å from its epoxide oxygen
- Corresponds to centroid part of baccatin ring system of the taxanes consisting of 1-OH, 9-carbonyl, and 15-gem-dimethyl group, which is 6.95 Å from its oxetane oxygen
- Common groups were then used as a template to fit the Epo molecule onto the baccatin ring system of taxanes

Results: Epo B Conformation Docked Into an Energy-refined Model of Tubulin



- Using previous pharmacophore and guided by the mutations in the Epo^R cells, docking Epo on an energy-refined model of the 3.7-Å density map of the docetaxel-binding site on β-tubulin is a success.
- Although, preference between the two Epo binding modes is not yet known.
  - Drug sensitivity data in 1A9 cells and PTX-selected cell line (PTX10), containing a $\beta 270^{Phe \rightarrow Val}$ mutation let the hypothesis be tested.

Results: Proving Epo Binding Mode II Is the Preferred Conformation

- Using an Epo B analogue with a pyridine moiety in place of a thiazole ring of the parent compound allowed binding mode II to be confirmed as the preferred conformation
  - Pyridine ring is bulkier than thiazole side chain, which in binding mode II is in close proximity to Phe-β270
  - A $\beta 270^{Phe \rightarrow Val}$ mutation would have a greater impact on sensitivity of pyridine-containing Epo B derivative compared with thiazole-containing molecule
  - Conformation is supported by cytotoxicity data previously

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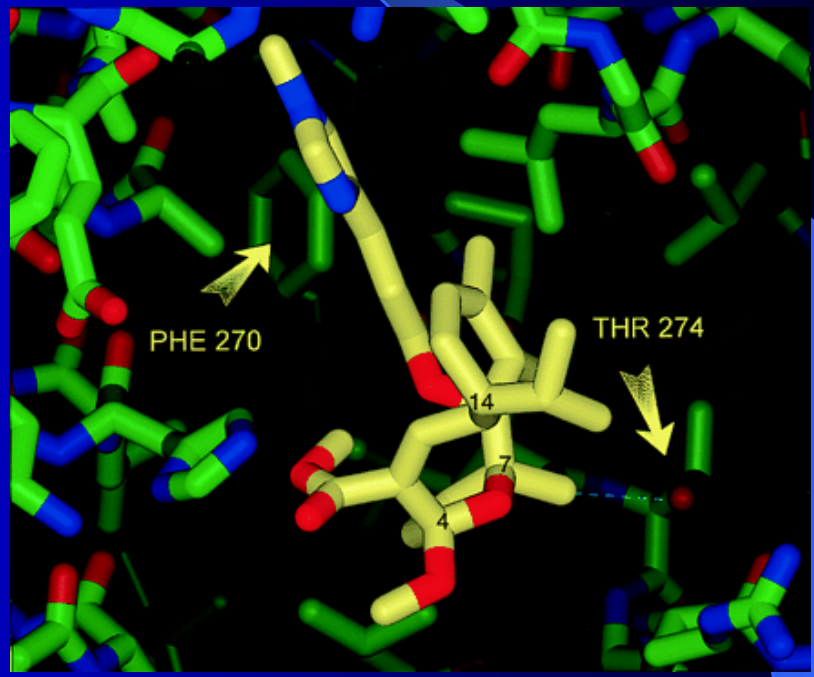
Sarcodictyins

- Will sarcodictyins fit into the pharmacophore between taxanes and epothilones???
  - Taxanes and epothilones have reduced activity in the mutant cells, but sarcodictyins' activity is enhanced
  - According to the pharmacophore, this can be explained

Sarcodictyin A Analogue Modeled Into the taxane Binding Site

- Same method used as with epothilones
  - Many similarities in structure are found between sarcodictyins and epothilones/taxanes
  - Specifically, the methyl group at C7 makes an unfavorable yet tolerable hydrophobic-polar interaction with the alcohol side chain of Thr-β274
  - In $\beta 274^{Thr \rightarrow Ile}$ mutant, the same interaction becomes a highly favorable hydrophobic interaction between the C7 methyl group and the Ile-β274 aliphatic side chain



Conclusion

- Success of taxanes makes tubulin an extremely attractive target for cancer chemotherapy
- Prompts research for compounds with similar functions
- Helps us understand interactions of these compounds with the common intracellular receptor, tubulin
- Data explained thus far sets the stage for advances in cancer chemotherapy

My Research Project…

- Discodermolide & Dictyostatin-1
  - Develop quantitative structural activity relationships (QSAR)
  - Model stabilizer bound to tubulin
  - Use cryoelectron microscopy

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Supplemental Slide: Tubulin Polymerization

- Tubulin: a globular protein that polymerized to form microtubules
- Polymerization: a chemical reaction in which two or more molecules combine to form larger molecules that contain repeating structural units

- Combine the two!

Supplemental Slide: Kinesin & Dynein

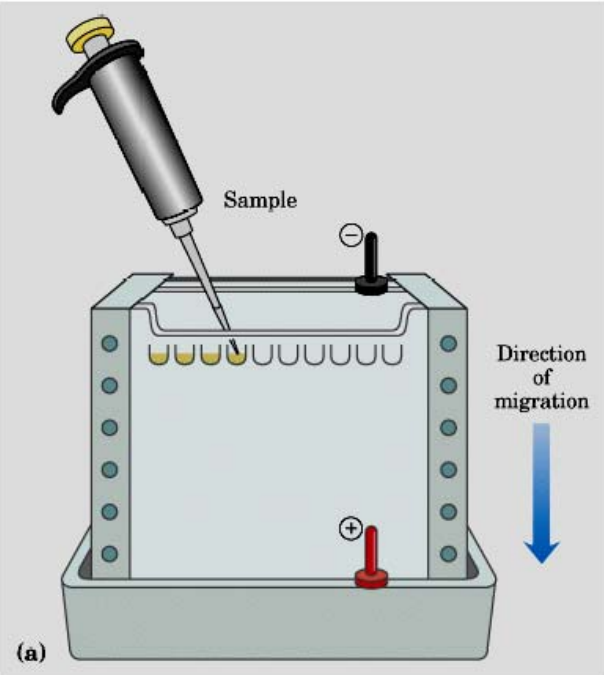
- Kinesin
  - Moves its associated cargo (vesicles and RNA protein particles) out along the microtubule network radiating from the centrosome
- Dynein
  - Moves its cargo towards the cell center

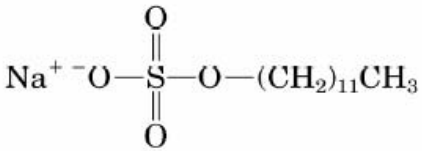
- Together, they form a two way transport system in the cell that is well developed in the axons and dendrites of nerve cells
- During mitosis, the cell assembles a mitotic apparatus of highly dynamic microtubules and uses microtubule motor proteins to separate the chromosomes into the daughter cells.

Supplemental Slide: Taxanes & Nontaxanes

- Taxanes: classes of natural products which inhibit cell growth by stopping cell division and contain baccatin
  - Also called mitotic inhibitors, antimitotic agents, antimicrotubule agents, etc.
- Nontaxanes: microtubule stabilizers which also inhibit cell growth, but vary in structure compared to taxanes

Supplemental Slide: Electrophoresis





Sodium dodecyl sulfate (SDS)

Electrophoresis is based on the migration of proteins in a charged field.

The force moving the macromolecules is the electrical potential, **E**.

μ = V/E, where μ is the electrophoretic mobility, and V is the velocity of the particle.

μ = Z/f, where **Z** is the net charge of the molecule, and **f** is the frictional coefficient. f is related to the the shape of the molecule, as well as its size.

Typically, the cross-linked polymer, polyacrylamide acts as the solid support. Normally, proteins would be separated in proportion to their charge-to mass ratio. The problem is that some proteins would migrate towards the anode, while others would migrate towards the cathode; and, the migration would not reflect size, but charge to mass.

The trick is to carry out the electrophoresis in the presence of sodium dodecyl sulfate, which is a detergent.

SDS binds to every protein in roughly the same proportion, which is about one molecule for every two amino acid residues. SDS carries with it a negative charge, and the cumulative negative charge renders the intrinsic net charge of the protein insignificant.

Therefore, every protein will have the same charge to mass ratio, which will cause all proteins to migrate towards the cathode with a rate that is dependent only on their sizes.

In contrast to gel-filtration, smaller molecules migrate faster than larger molecules because they can snake through the pores of the gel better.

Typically, this technique is not used to purify proteins, because SDS normally denatures proteins. It is used to analyze the purity of proteins.