Iron

From Current Biochemistry to New Chelator Development Strategies

September 21-22, 1998

Lister Hill Auditorium

National Institutes of Health

Bethesda, Maryland

Acknowledgements

The organizers would like to thank their many colleagues for assistance in planning this meeting. These include the planning committee listed below, and in particular, Dr Ray Bergeron and Dr. Gary Brittenham, who spent many hours in developing the program. Thanks also go to the co-sponsors of this meeting, the Cooley's Anemia Foundation, Inc., the National Heart, Lung and Blood Institute, Novartis Pharmaceuticals Corporation, and SunPharm Corporation.

Planning Committee

Philip Aisen, MD
Nancy C. Andrews, PhD
Raymond J. Bergeron, PhD
Gary M. Brittenham, MD
John W. Eaton, PhD
Chaim Hershko, MD
David G. Nathan, MD
Professor Sir David J. Weatherall

Josephine P. Briggs, MD
Director
Division of Kidney, Urologic and Hematologic Diseases
National Institute of Diabetes and Digestive and Kidney Diseases

David G. Badman, PhD
Hematology Program Director
Division of Kidney, Urologic and Hematologic Diseases
National Institute of Diabetes and Digestive and Kidney Diseases

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Iron

From Current Biochemistry to New Chelator Development Strategies

Lister Hill Auditorium, NIH Campus
Pooks Hill Marriott
Bethesda, Maryland
September 21-22, 1998

Monday, September 21, 1998

7:30 am–8:30 am	Registration/Continental Breakfast	
8:30 am–8:40 am	Introduction: Overview and Purpose of the Workshop	*David G. Badman, PhD*
	Session I— *Advances in Iron Biochemistry and Biology*	
	Fenton Chemistry and Mechanisms of Iron Toxicity *Chair: Raymond J. Bergeron, PhD*	
8:40 am–9:05 am	Kinetics and Mechanism of the Fenton Reaction. Implications of Iron Toxicity	*Willem H. Koppenol, PhD*
9:05 am–9:30 am	Iron-driven Damage to HbSS and Thalassemic Erythrocytes	*Robert P. Hebbel, MD*
9:30 am–9:55 am	Intracellular Iron and Cell Death	*Ulf Brunk, MD, PhD*
9:55 – 10:15 am	*Break*	
10:15 am–10:35am	Chelators and Liver Disease/NF-kB Activation	*Hidekazu Tsukamoto, PhD*
10:35 am–11:05 am	Iron, DNA and Chromatin Remodeling	*Sally A. Leong, PhD*

11:05 am–11:35 am	Mechanism of Hemopexin— Mediated Protection Against Oxidative Reactions	*Ann Smith, PhD*
11:35 am–11:50 am	*Panel Discussion*	
11:50 am–1:00 pm	*Lunch (on own)*	

Basic Biology of Iron Transport
Chair: Philip Aisen, MD

1:00 pm–1:30 pm	Critical Steps in Transmembrane Iron Transport	*Nancy Andrews, MD, PhD*
1:30 pm–2:00 pm	Mitochondrial Iron Transport; Yeast Genetics	*Jerry Kaplan, PhD*
2:00 pm–2:30 pm	Metabolism and Transport of Iron: Distinct Control Mechanisms in Erythroid Cells	*Premsyl Ponka, MD, PhD*
2:30 pm–3:00 pm	*Break*	

Regulation of Cellular Iron Metabolism in Humans
Chair: Nancy C. Andrews, MD, PhD

3:00 pm–3:25 pm	Regulation of Erythropoiesis	*H. Franklin Bunn, MD*
3:25 pm–3:50 pm	The Role of Iron Regulatory Proteins in Maintenance of Iron Homeostasis in Mammals	*Tracey Rouault, MD*
3:50 pm–4:10 pm	Cellular Aspects of Ferritin Metabolism	*Kenneth R. Bridges, MD*
4:10 pm–4:30 pm	A New Twist in Ferritin Iron Release	*Elizabeth C. Theil, PhD*
4:30 pm–5:00 pm	*Panel Discussion*	
6:30 pm–9:30 pm	Dinner/Speaker—Pooks Hill Marriott The Mining of Iron Lore	*David G. Nathan, MD*

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Tuesday, September 22, 1998

7:30 am–8:00 am	Registration/Continental Breakfast	
	Session II— *Iron Chelator Design for the Treatment of Iron Overload*	
	Chair: David G. Badman, PhD	
8:00 am–8:30 am	Overview of the Current Status of Chelation Therapy	*Chaim Hershko, MD*
8:30 am–9:00 am	Introduction to Chelator Design	*Kenneth N. Raymond, PhD*
	Mechanisms of Iron Chelator Toxicity *Chair: Chaim Hershko, MD*	
9:00 am–9:30 am	Clinical Toxicity of Iron Chelators	*Beatrice E. Gee, MD*
9:30 am–10:00 am	Clinical Effectiveness of Chelation Therapy	*Nancy Olivieri, MD, FRCP*
10:00 am–10:20am	*Break*	
	Alternative Approaches for the Management of Iron Overload	
10:20 am–10:40am	Transfusional Approaches to the Management of Iron Overload	*Alan Cohen, MD*
10:40 am–11:10 am	Alternative Approaches to Chelator Development	*Raymond J. Bergeron, PhD*
11:10 am–11:30pm	Iron Chelation: Rationale for Combination Therapy	*Robert W. Grady, PhD*
11:30 am–12:45 pm	*Lunch (on own)*	
	Drug Development *Chair: Alan Cohen, MD*	
12:45 pm–1:05 pm	Preclinical Evaluation of ICL670A— A Novel Orally Active Iron Chelator	*Hans Peter Schnebli, PhD*
1:05 pm–1:25 pm	Novartis Chelators Program— Clinical Development Update	*Daniele Alberti, MD*

1:25 pm–1:45 pm	Update on Depot Desferrioxamine; Implications for Chelation Regimens	*John B. Porter, MA, FRCP*
1:45 pm–2:30 pm	*Roundtable Discussion: NIH Participation in Drug Development— How can it be most Effective?*	
	Body Iron Measurement: Effects of Chelators on Body Iron Distribution	
2:30 pm–2:50 pm	New Approaches to the Non-Invasive Measurement of Iron Overload	*Gary M.Brittenham, MD*
	Iron Chelator Evaluation for the Treatment of Iron Overload and Other Potential Applications of Iron Chelators *Chair: Gary M. Brittenham, MD*	
	Novel Methods for Pre-Clinical Evaluation of Candidate Iron Chelating Agents	
2:50 pm–3:10 pm	Viewing and Accessing the Cellular Iron Pools	*Z. Ioav Cabantchik, MD, PhD*
3:10 pm–3:30 pm	Toxicological Evaluation of Iron Chelators	*Philip Carthew, PhD*
3:30 pm–3:45 pm	*Break*	
	Iron Chelators in Therapeutic Strategies for the Treatment of Other Diseases	
3:45 pm–4:05 pm	Sickle Cell Anemia	*Elliott Vichinsky, MD*
4:05 pm–4:25 pm	Reperfusion Injury; Diabetes	*John W. Eaton, PhD*
4:25 pm–4:45 pm	Exacerbation of Fungal Infections by Siderophores; Role of Iron in HIV	*Johan R. Boelaert, MD*
4:45 pm–5:05 pm	Malaria	*Victor R. Gordeuk, MD*
5:05 pm–5:30 pm	Closing Remarks: Problems and Perspectives	*Philip Aisen, MD*
	ADJOURNED	

Planning Committee

Philip Aisen, MD
Professor
Department of Physiology and Biophysics
Albert Einstein College of Medicine
1300 Morris Park Avenue
Bronx, NY 10461
718-430-2593
718-430-8819
aisen@aecom.yu.edu

Nancy C. Andrews, MD, PhD
Assistant Investigator
Howard Hughes Medical Institute
Children's Hospital, Harvard Medical School
300 Longwood Avenue, Enders 720
Boston, MA 02115
617-355-8183
617-734-6791
andrews_n@a1.tch.harvard.edu

David G. Badman, PhD
Hematology Program Director
Division of Kidney, Urologic and Hematologic Diseases
National Institute of Diabetes and Digestive and Kidney Diseases, NIH
Natcher Building, Room 6AS-13C
45 Center Drive
Bethesda, MD 20892
301-594-7717
301-480-3510
david_badman@nih.gov

Raymond J. Bergeron, PhD
Graduate Research Professor
Medicinal Chemistry
University of Florida
Box 100485 JHMHC
Gainesville, FL 32610-0485
352-846-1956
352-392-8406
bergeron@mc.cop.ufl.edu

Josephine P. Briggs, MD
Director, Division of Kidney, Urologic and Hematologic Diseases
National Institute of Diabetes and Digestive and Kidney Diseases
National Institutes of Health
Building 31, Room 9A-17
31 Center Drive, MSC 2560
Bethesda, MD 20892-2560
301-496-6325
301-402-4874
briggsj@hq.niddk.nih.gov

Gary M. Brittenham, MD
Professor of Medicine and Pediatrics
Departments of Medicine and Pediatrics/
Division of Hematology
Columbia University College of Physicians and Surgeons
530 West 168th Street
New York, NY 10032
212-305-6531
212-305-8408
gmb2@po.cwru.edu

Planning Committee

John W. Eaton, PhD
Professor of Pediatrics
Department of Pediatrics
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030
713-798-5466
713-790-0617
eatonredox@aol.com

Chaim Hershko, MD
Professor of Medicine
Hebrew University Medical School
Shaave Zedek Medical Center, PO Box 3235
Jerusalem, Israel 91031
972-2-6555567
972-2-6513946
hershko@szmc.org.il

David G. Nathan, MD
President
Dana-Farber Cancer Institute
44 Binney Street
Boston, MA 02115
617-632-2155
617-632-2161
david_nathan@dfci.harvard.edu

Premsyl Ponka, MD, PhD
Professor
Lady Davis Institute — Jewish General Hospital
McGill University
3755 Cote Ste. Catherine Road
Montreal, Quebec H3T 1E2
Canada
514-340-8260
514-340-7502
mdpp@musica.mcgill.ca

Speakers

Philip Aisen, MD
Professor
Department of Physiology and Biophysics
Albert Einstein College of Medicine
1300 Morris Park Avenue
Bronx, NY 10461
718-430-2593
718-430-8819
aisen@aecom.yu.edu

Daniele Alberti, MD
International Clinical Leader
Medical Department
Novartis Farma S.P.A
SS 233 (Varesina) km 20,5
Origgio, Italy (VA) 21040
0039-02-96542817
0039-02-96543476
daniele.alberti@pharma.novartis.com

Nancy C. Andrews, MD, PhD
Assistant Investigator
Howard Hughes Medical Institute
Children's Hospital, Harvard Medical School
300 Longwood Avenue, Enders 720
Boston, MA 02115
617-355-8183
617-734-6791
andrews_n@a1.tch.harvard.edu

Raymond J. Bergeron, PhD
Graduate Research Professor
Medicinal Chemistry
University of Florida
Box 100485 JHMHC
Gainesville, FL 32610-0485
352-846-1956
352-392-8406
bergeron@mc.cop.ufl.edu

Johan R. Boelaert, MD
Director of Department of Infectious Diseases
Unit of Renal and Infectious Diseases
Algemeen Ziekenhuis St.-Jan
Ruddershove, 10
Brugge, B-8000
Belgium
32-50-452200
32-50-452299
j.r.boelaert.brugge@unicall.be

Kenneth R. Bridges, MD
Director, Joint Center for Sickle Cell and Thalassemic Disorders
Brigham & Women's Hospital
BLI, Room 327
221 Longwood Avenue
Boston, MA 02115
617-732-5842
617-975-0876
bridges@clavin.harvard.edu

Gary M. Brittenham, MD
Professor of Medicine and Pediatrics
Departments of Medicine and Pediatrics/
Division of Hematology
Columbia University College of Physicians
and Surgeons
530 West 168th Street
New York, NY 10032
212-305-6531
212-305-8408
gmb2@po.cwru.edu

Ulf Brunk, MD, PhD
Professor of Pathology
Department of Pathology
University of Linhoping
Linkoping, Sweden S-581 85
46-13-221515
46-13-221529
ulf.brunk@pat.liu.se

Speakers

H. Franklin Bunn, MD
Professor of Medicine
Harvard Medical School
Room 223 LMRC
221 Longwood Avenue
Boston, MA 02115
617-732-5841
617-739-0748
bunn@calvin.bwh.harvard.edu

Z. Ioav Cabantchik, MD, PhD
Professor of Biochemistry and Biophysics
Department of Biological Chemistry
Hebrew University of Jerusalem
Givat Ram, Jerusalem, Israel 91904
972-2-658-5420
972-2-658-6974
ioav@cc.huji.ac.il

Philip Carthew, PhD
Senior Scientist
Medical Research Council Toxicology Unit
University of Leicester
Hodgkin Building
Lancaster Road
Leicester, United Kingdom LEI 9HN
44-0116-2525608
44-0116-2525616
pc13@le.ac.uk

Alan Cohen, MD
Chief, Division of Hematology
Children's Hospital of Philadelphia
34th Street and Civic Center Boulevard
Philadelphia, PA 19104
215-590-3438
215-590-3525
cohen@email.chop.edu

John W. Eaton, PhD
Professor of Pediatrics
Department of Pediatrics
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030
713-798-5466
713-790-0617
eatonredox@aol.com

Beatrice E. Gee, MD
Assistant Professor
Morehouse School of Medicine
1825 Meadowdale Avenue, NE
Atlanta, GA 30306
404-872-5144
404-756-1357
jpmontoya@earthlink.net

Victor R. Gordeuk, MD
Associate Professor of Medicine
Department of Medicine,
Division of Hematology and Oncology
The George Washington University
Medical Center
Suite 3-428
2150 Pennsylvania Avenue, NW
Washington, DC 20037
202-994-3556
202-994-4524
domvrg@gwumc.edu

Robert W. Grady, PhD
Associate Research Professor
Department of Pediatrics
Cornell University Medical College
Room S-600B
515 East 71st Street
New York, NY 10021
212-746-3422
212-746-8609
rwgrady@mail.med.cornell.edu

Speakers

Robert P. Hebbel, MD
Professor and Vice-Chairman
Department of Medicine
University of Minnesota Medical School
Box 480 UMHC
Harvard Street at East River Road
Minneapolis, MN 55455
612-624-6104
612-625-6919
hebbe001@tc.umn.edu

Chaim Hershko, MD
Professor of Medicine
Hebrew University Medical School
Shaave Zedek Medical Center, PO Box 3235
Jerusalem, Israel 91031
972-2-6555567
972-2-6513946
hershko@szmc.org.il

Jerry Kaplan, PhD
Professor of Pathology
Department of Pathology
University of Utah
5C130 SOM
50 North Medical Drive
Salt Lake City, UT 84132
801-581-7427
801-581-4517
kaplan@bioscience.biology.utah.edu

Willem Hendrik Koppenol, PhD
Professor
Laboratory for Inorganic Chemistry
ETH Zurich
Universitatstr. 6
Zurich, Switzerland CH-8092
4101-632-28-75
4101-632-10-90
koppenol@inorg.chem.ethz.ch

Sally Ann Leong, PhD
Research Chemist and Professor
Department of Plant Pathology
USDA, ARS and University of Wisconsin
1630 Linden Drive
Madison, WI 53706
608-262-5309
608-262-1541
sal@plantpath.wisc.edu

David G. Nathan, MD
President
Dana-Farber Cancer Institute
44 Binney Street
Boston, MA 02115
617-632-2155
617-632-2161
david_nathan@dfci.harvard.edu

Nancy Olivieri, MD, FRCP(C)
Professor, Pediatrics and Medicine
University of Toronto
The Hospital for Sick Children
Room 9413
555 University Avenue
Toronto, Canada M5G 1X8
416-813-6823
416-813-5346
noliv@sickkids.on.ca

Premsyl Ponka, MD, PhD
Professor
Lady Davis Institute—Jewish General Hospital
McGill University
3755 Cote Ste. Catherine Road
Montreal, Quebec, Canada H3T 1E2
514-340-8260
514-340-7502
mdpp@musica.mcgill.ca

Speakers

John B. Porter, MA, FRCP, FRCPath.
Department of Haematology
University College London
98 Chenies Mews
London, England WC1E 6HX
United Kingdom
0171-209-6224
0171-209-6222
j.porter@ucl.uc.uk

Kenneth N. Raymond, PhD
Professor
Department of Chemistry
University of California, Berkeley
Berkeley, CA 94720-1460
510-642-7219
510-486-5283
raymond@socrates.berkeley.edu

Tracey Rouault, MD
Chief, Section on Human Iron Metabolism
Cell Biology and Metabolism Branch
National Institute of Child Health and Human Development, NIH
Building 18T, Room 101
Bethesda, MD 20892
301-402-3428
301-402-0078
trou@helix.nih.gov

Hans Peter Schnebli, PhD
Head Iron Chelation Research
Novartis Pharma
K-125.120
Basel, Switzerland CH-4002
0041-61-696-71-94
0041-61-696-76-60
hans_peter.schnebli@pharma.novartis.com

Ann Smith, PhD
Professor
Division of Molecular Biology and Biochemistry
University of Missouri Kansas City
School of Biological Sciences
5007 Rockhill Road
Kansas City, MO 64110
816-235-2579
816-235-5595
annsmith@cctr.umkc.edu

Elizabeth C. Theil, PhD
Research Scientist
Research Institute
Children's Hospital Oakland
747 52nd Street
Oakland, CA 94609
510-428-3775
510-597-7131
etheil@mail.cho.org

Hidekazu Tsukamoto, PhD
Professor of Medicine and Pathology
University of Southern California
School of Medicine
HMR-101
2011 Zonal Avenue
Los Angeles, CA 90033-1034
323-442-5107
323-442-5425
htsukamo@hsc.usc.edu

Elliott Vichinsky, MD
Director, Hematology/Oncology
Children's Hospital Oakland
747 52nd Street
Oakland, CA 94609
510-428-3651
510-450-5647
evichinsky@lanminds.com

Participants

Charles Agius, PhD
Assistant Director, Oncology New Products
Novartis Pharmaceudicals Corporation
59 Route 10
East Hanover, NJ 07936-1080
973-781-3095
973-781-6555
charles.agius@pharma.novartis.com

Randy S. Alexander
Chairman and President
Iron Disorders Institute
P.O. Box 2031
Greenville, SC 29602
864-292-1175
864-878-5594
randyhgi@mci2000.com

Charles Allerson, PhD
IRTA Postdoctoral Fellow
National Institute of Child Health and
Human Development, NIH
Building 18T, Room 101
9000 Rockville Pike
Bethesda, MD 20892
301-402-0360
301-402-0078
allerson@box-a.nih.gov

Steven D. Aust, PhD
Professor
Utah State University
Biotechnology Center
Logan, UT 84322-4705
435-797-2730
435-797-2755
sdaust@cc.usu.edu

William Avrin, PhD
Contractor in Applied Physics
Quantum Magnetics
7740 Kenamar Court
San Diego, CA 92121
619-566-9200
619-566-9388
bavrin@ucsd.edu

James K. Bashkin, PhD
Assistant Professor of Chemistry
Department of Chemistry
Washington University in St. Louis
Campus Box 1134
One Brookings Drive
St. Louis, Missouri 63130-4899
314-935-4801
314-935-4481
bashkin@wuchem.wustl.edu

David Brooks, MD, PhD
Fellow, Division of Medical Genetics
Hospital of the University of Pennsylvania
1 Maloney Building
34th & Spruce Street
Philadelphia, PA 19104
215-662-4740
215-614-0298
brooksda@mail.med.upenn.edu

Jack J. Buchcese
President and CEO
CytRx Corporation
154 Technology Parkway
Norcross, GA 30092
770-368-9500
770-448-3357
buchesej@cytrx.com

Participants

Joan L. Buss, PhD
Postdoctoral Fellow
Physiology Department, McGill University
Lady Davis Institute for Medical Research
Sir Mortimer B. Davis Jewish General Hospital
Montreal, Quebec, Canada H3T 1E2
514-340-8222 x 4666
514-340-7502
ezcq@musica.mcgill.ca

Alison Butler, PhD
Professor
Department of Chemistry
University of California
Santa Barbara, CA 93106-9510
805-893-8178
805-893-4120
butler@chem.ucsb.edu

N. Dennis Chesteem, PhD
Professor
Department of Chemistry
University of New Hampshire
Parsons Hall
Durham, NH 03824
603-862-2520
603-862-4278
ndc@cisunix.unh.edu

Allen Clarkson, PhD
Associate Professor
New York University School of Medicine
341 East 25th Street
New York, NY 10010
212-263-6637
212-263-6637
allen.clarkson@med.nyu.edu

James Connor, PhD
Professor
Department of Neuroscience and Anatomy
Penn State University
M.S. Hershey Medical Center
Hershey, PA 17033
717-531-6903
717-531-5184
jrc3@psu.edu

Bill Crowe, PhD
Research Assistant Professor
Department of Physiology
Allegheny University Health Sciences
2900 Queen Lane
Philadelphia, PA 19129
215-991-8418
215-843-6516
crowe@auhs.edu

Alvin L. Crumbliss, PhD
Professor of Chemistry
Chemistry Department
Duke University
Box 90346
Durham, NC 27708-0346
919-660-1540
919-660-1605
alc@chem.duke.edu

Harry A. Dailey, PhD
Professor
Department of Biochemistry and
Molecular Biology
University of Georgia
Athens, GA 30602-2605
706-542-2690
706-542-7567
dailey@bscr.uga.edu

Participants

Sylvain Dore, PhD
Post Ph.D. Fellow
Department of Neuroscience
Johns Hopkins University/School of Medicine
725 North Wolfe Street
Baltimore, MD 21205
410-955-3083
410-955-3623
sdore@welchlinte.welch.jhu.edu

Steve Drake, PhD
IRTA
Cell Biology and Metabolism Branch
National Institute of Child Health and Human Development, NIH
Building 18T, Room 101
Bethesda, MD 20892
301-402-0358
301-402-0078
drake@boxd-nih.gov

Eileen Eiler, PhD
Scientific Writer
Medicinal Chemistry Department
University of Florida
Room P-401
1600 S.W. Archer Road, P.O. Box 100485
Gainesville, FL 32610
352-846-1956
352-392-8406

R. Martin Emanuele, PhD
Vice President,
Business Development and Research
CytRx Corporation
154 Technology Parkway
Norcross, GA 30092
770-368-9500
770-448-3357
emanuele@cytrx.com

Christopher D. Ferris, MD, PhD
Fellow
Department of Neuroscience
The Johns Hopkins University School of Medicine
725 N. Wolfe Street
Baltimore, MD 21205
410-955-3024
410-955-3623
cdferris@welchlink.welch.jhu.edu

Roland Fischer, PhD
Senior Physicist
UKE—Physiol. Chem. Institute
Martinistr.52
Hamburg, Germany D-20246
49-40-4717-3385
49-40-4717-4797
fischer@uke.uni-hamburg.de

Patricia J. Giardina, MD
Associate Professor of Pediatrics
Chief Division Pediatric Hemetology/Oncology
New York Hospital Cornell Medical Center
525 East 68th Street
New York, NY 10021
212-746-3415
212-746-8609

Hilary A. Godwin, PhD
Assistant Professor
Department of Chemistry
Northwestern University
2145 Sheridan Road
Evanston, IL 60208
847-467-3543
847-491-7713
hagodwin@chem.nwu.edu

Participants

J. Michael Grindel, PhD
Vice President, Drug Development
CytRx Corporation
154 Technology Parkway
Norcross, GA 30092
770-453-0120
770-448-3357
grindelm@cytrx.com

Hiromi Gushin, PhD
Research Fellow
The Children's Hospital
300 Longwood Avenue
Boston, MA 02115
617-355-5413
617-734-6791
gushin_h@al.tch.harvard.edu

Carlos G. Gutierrez, PhD
Professor of Chemistry
Department of Chemistry and Biochemistry
California State Universtiy
5151 State University Drive
Los Angeles, CA 90032
213-343-2356
213-343-6411
cgutier@calstatela.edu

Paul Harmatz, MD
Pediatric - Gastroenterologist
Children's Hospital Oakland
747 52nd Street
Oakland, CA 94609
510-254-0724
510-450-5813
pharmatz@aol.com

Mary Harris
Writer-Editor
Office of Scientific and Health Information
National Institute of Diabetes and Digestive
and Kidney Diseases, NIH
Building 31, Room 9A04
Bethesda, MD 20892
301-435-8114
301-496-7422
mary_harris@nih.gov

Bo E. Hedlund, PhD
Chief Scientific Officer
Biomedical Frontiers, Inc.
1095 10th Avenue, S.E.
Minneapolis, MN 55414
612-378-0228
612-378-3601

R.C. Hider, PhD
Professor of Medicinal Chemistry
Department of Pharmacy
Kings College London
Manresa Road
London, SW36LX
United Kingdom
0171-333-4646
0171-937-5690
robert.hides@kcl.ac.uk.

Nathan C. Ihle, PhD
Director of Pharmaceutical Chemistry
PathoGenesis Corporation
201 Elliott Avenue West, Suite 150
Seattle, WA 98119
206-674-6686
206-282-5065
nihle@pathogenesis.com

Participants

Amanda L. Jenkins, PhD
Associate Professional Chemist
Johns Hopkins Applied Physics Lab
Building 13 N250
11100 Johns Hopkins Road
Laurel, MD 20723-6099
443-778-8631
443-778-6914
jenkial1@aplmsg.jhuapl.edu

J. Shawn Jones, PhD
Graduate Assistant
Pharmacology and Toxicology Department
University of Texas Medical Branch at Galveston
10th and Market Streets
Galveston, TX 77555-1031
409-772-1561
409-772-9642
sjones@marlin.utmb.edu

Kashi Karimian, PhD
Head, Medicinal Chemistry
Apotex Inc.
150 Signet Drive
Weston, Ontario M9L1T9
Canada
416-401-7303
416-401-3809
kkarimia@apotex.ca

John Kemp, MD
Professor of Pathology
University of Iowa College of Medicine
University of Iowa Hospitals
5238 Carver Pavilion
Iowa City, Iowa 52242
319-356-4811
319-356-8470
john-kemp@uiowa.edu

John Ketchum
Director, Oncology New Products
Novartis Pharmaceuticals Corporation
59 Route 10
East Hanover, NJ 07936-1080
973-781-6106
973-781-6555
john.ketchum@pharma.novartis.com

Gopal Krishna, PhD
Emeritus Scientist
National Institutes of Mental Health, NIH
Building 10 - 3DL11
301-496-3421
301-402-0188
krishna@helix.nih.gov

Sankaran Kumar, PhD
Senior Scientist
Quantum Magnetics, Inc.
7740 Kenamar Court
San Diego, CA 92121
619-566-9200
619-566-9388
kumar@qm.com

Susan S. Kurth, MPH
Nurse Practitioner for Sickle Cell and Thalassemia
Childrens Hospital Boston
Bader 403
300 Longwood Avenue
Boston, MA 02115
617-355-8304
617-738-9857
kurth@a1.tch.harvard.edu

Participants

Tiit Land, PhD
Postdoctoral
Cell Biology and Metabolism Branch
National Instiutes of Child Health and
Human Development, NIH
Building 18T/101
9000 Rockville Pike
Bethesda, MD 20814
301-402-0358
301-402-0078
tiit@box-t.nih.gov

Timothy LaVaurte
Research Associate
Cell Biology and Metabolism Branch
National Institute of Child Health and
Human Development, NIH
Building 18T, Room 101
9000 Rockville Pike
Bethesda, MD 20892
301-402-0359
301-402-0078
tlavaurte@box-t.nih.gov

Carol H. Letendre, PhD
Deputy Director
Division of Blood Diseases and Resources
National Heart, Lung, and Blood Institute, NIH
Rockledge II, Room 10162
6701 Rockledge Drive, MSC 7950
Bethesda, MD 20892-7950
301-435-0080
301-480-0867
letendrc@gwgate.nhlbi.nih.gov

Joanne Levy, MD
Instructor in Medicine
Brigham and Women's Hospital
Enders 708, Children's Hospital
320 Longwood Avenue
Boston, MA 02115
617-355-7474
617-734-6791
jelevy@bics.bwtt.harvard.edu

Gang Liu, PhD
Research Associate
University of Utah
40 N 2030 E Front
Salt Lake City, UT 84112-5860
801-585-1460
801-581-7008
gang.liu@m.cc.utah.edu

Filomena Longo, MD
Postgraduate
Department of Pediatrics University of Toronto
Thalassemia Centre
Piazza Polonia 94
Torino, Italy 10126
39-11-3135291
39-11-3135309
longo@pediatria.unito.it

Mark Loyevsky, PhD
Research Associate
Department of Medicine,
Division of Hematology/ Oncology
George Washington University
2150 Pennsylvania Avenue, NW
Washington, DC 20037
202-994-7482
202-944-4524
dommxe@gwumc.edu

Participants

Jack J. Luchese
President and CEO
CytRx Corporation
154 Technology Parkway
Norcross, GA 30092
770-368-9500
770-448-3357
luchesej@cytrx.com

Lilly Maglova, PhD
Research Associate
Department of Physiology
Allegheny University Health Sciences
2900 Queen Lane
Philadelphia, PA 19129
215-991-8419
215-843-6516
maglova@auhs.edu

Arthur E. Martell, PhD
Distinguished Professor
Department of Chemistry
Texas A&M University
P.O. Box 300012
College Station, TX 77842-3012
409-845-5055
409-845-4719
martell@mail.chem.tamu.edu

David P. Mascotti, PhD
Assistant Professor
Department of Chemistry
John Carroll University
20700 North Park Boulevard
University Heights, OH 44118
216-397-4241
216-397-3033
dmascotti@jcvaxa.jcu.edu

Douglas McAbee, PhD
Assistant Professor of Biochemistry
Department of Chemistry and Biochemistry
California State University Long Beach
1250 Bellflower Boulevard
Long Beach, CA 90840
562-985-1558
562-985-8557
dmcabee@csulb.edu

Gordon D. McLaren, MD
Associate Professor of Medicine
University of California, Irvine
VA Medical Center (11/111-H)
5901 East Seventh Street
Long Beach, CA 90822
562-494-5526
562-494-5456
mclaren.gordon@long-beach.va.gov

James McManis, PhD
Postdoctoral Associate
Medicinal Chemistry Department
University of Florida
Room P-401
1600 S.W. Archer Road, P.O. Box 100485
Gainsville, FL 32610
352-846-1956
352-392-8406

Salim Merali, PhD
Assistant Professor
New York University Medical Center
341 East 25th Street
New York, NY 10010
212-263-8499
212-263-6637
merals01@popmail.med.nyu.edu

Participants

Esther Meyron-Holtz, PhD
Visiting Fellow
Cell Biology and Metabolism Branch
National Instiutes of Child Health and
Human Development, NIH
Building 18T, Room 101
Bethesda, MD 20892
301-402-0358
301-402-0078
meyron@box_m.nih.gov

Marvin J. Miller, PhD
George and Winifred Clark Professor of Chemistry
Department of Chemistry and Biochemistry
University of Notre Dame
251 Nieuwland Science Hall
Notre Dame, IN 46556-5670
219-631-7571
219-631-6652
marvin.j.miller.2@nd.edu

Celine Moore, PhD
Manager, Preclinical Evaluation
Hemosol Inc.
2 Meridian Road
Toronto, Ontario M9W 4Z7
Canada
416-798-0700
416-798-0057
cmoore@hemosol.com

Werner Niederberger, PhD
Project Team Representative
Novartis Pharma AG
K-135.1.71
Basel, Switzerland CH-4002
41-61-696-87-43
41-61-696-69-92
werner.niederberger@pharma.novartis.com

Peter Nielsen, MD, PhD
Physician Chemist
UKE—Physiol. Chem. Institute
Martinistr.52
Hamburg, Germany 20246
49-40-4717-3389
49-40-4717-4797
nielsen@uke.uni-hamburg.de

Eric Nisbet-Brown, MD, PhD
Medical Director, Thalassemia Program
Children's Hospital Boston
Bader 403
300 Longwood Avenue
Boston, MA 02115
617-355-2457
617-738-9857
nisbet_e@a1.tch.harvard.edu

Constance Tom Noguchi, PhD
Section Chief
Laboratory of Chemical Biology
National Institute of Diabetes and Digestive
and Kidney Diseases, NIH
Building 10, 9N307
10 Center Drive MSC-1822
Bethesda, MD 20892-1822
301-496-1163
301-402-0101
cnoguchi@helix.nih.gov

S. Scott Panter, PhD
Associate Research Scientist
Department of Neurosurgery, UCSF
VAMC, Neurology (127)
4150 Clement Street
San Francisco, CA 94121
415-221-4810 x 3964
415-750-2273
panter.s_scott@sanfrancisco.va.gov

Participants

Caroline Philpott, MD
Tenure-Track Investigator
Liver Diseases Section
National Institute of Diabetes and Digestive
and Kidney Diseases, NIH
Building 10, Room 9B16
10 Center Drive, MSC 1800
Bethesda, MD 20892-1800
301-435-4018
301-402-0491
carolinep@intra.niddk.nih.gov

Antonio Piga, MD
Head Thalassemia Centre
Department of Pediatrics
University of Torino
Piazza Polonia 94
Torino, Italy 10126
39-11-3135291
39-11-3135309
piga@pediatria.unito.it

Roy P. Planalp, PhD
Associate Professor of Chemistry
Department of Chemistry
University of New Hampshire
Parsons Hall
Durham, NH 03824
603-862-2471
603-862-4278
roy.planalp@unh.edu

Pensri Pootrakul, MD
Professor
Division of Hematology, Department of Medicine
Mahidol University
Thalassemia Research Center
Siriras Hospital
Bangkok, Thailand 10700
662-889-2557
662-889-2559
grppt@mahidol.ac.th

Jerry Retkwa, RPH, MS
Senior Clinical Research Scientist
Novartis Pharmaceuticals
59 Route 10
East Hanover, NJ 07936-1080
973-781-7042
973-781-5511
jerry.rctkwa@pharma.novartis.com

Des Richardson, PhD
Research Fellow
Department of Medicine
University of Queensland, Australia
C Floor, Clinical Sciences Building
Royal Brisbane Hospital
Herston, Brisbane, Australia 4029
61-7-3365-5121
61-7-3365-5462
d.richardson@medicine.herston.uq.edu.au

Eileen A. Ryan
Associate Director, Drug Regulatory Affairs
Novartis Pharmaceuticals Corporation
59 Route 10
East Hanover, NJ 07936
973-781-7661
973-781-6325
eileen.ryan@pharma.novartis.com

Peter Sinclair, PhD
Research Biologist
VA/Dartmouth Medical School
VA (151)
White River Junction, VT 05009
802-295-9363 x 5657
802-296-6308
psinc@dartmouth.edu

Participants

Sophia R. Smith, MD
Clinical Fellow
Cell Biology and Metabolism Branch
National Institutes of Child Health and
Human Development, NIH
Building 18T, Room101
18 Library Drive
Bethesda, MD 20892
301-496-6368
301-402-0078
sophies@helix.nih.gov

Michael Spino, PhD
Senior Vice President, Scientific Affairs
Apotex Inc.
150 Signet Drive
Weston, Ontario, Canada M9L 1T9
416-401-7280
416-401-3878
mspino@apotex.ca

Somdet Srichairatanakool
Department of Haematology
University College London
Faculty of Clinical Science
London, England WC1E6HX
0044-171-2096230
0044-171-2096222
rmgvssr@ucl.ac.uk

Richard R. Streiff, MD
Professor of Medicine
University of Florida College of Medicine
P.O.Box 100277, Archer Road
Gainsville, FL 32610
352-374-6018
352-374-6116

Michael B. Streiff, MD
Instructor in Medicine
Johns Hopkins University School of Medicine
Ross Building, Room 1025
720 Rutland Avenue
Baltimore, MD 21205
410-614-0727
410-955-0185
mstreif@welchlink.welch.jhv.edu

Wing-Hang Tong, PhD
Visiting Fellow
Cell Biology and Metabolism Branch
National Institute of Child Health and
Human Development, NIH
Bethesda, MD 20892
301-402-0358
301-402-0078
wing@box-w.nih.gov

Richard W. Topham, PhD
Professor of Chemistry
Department of Chemistry
University of Richmond
Gottwald Science Center
Richmond, VA 23173
804-289-8244
804-289-8482
rtopham@richmond.edu

Frank M. Torti, MD
Director, Comprehensive Cancer Center;
Chair, Department of Cancer Biology
Comprehensive Cancer Center of
Wake Forest University
Wake Forest University School of Medicine
Medical Center Boulevard
Winston-Salem, NC 27157-1082
336-716-7971
336-716-0293
ftorti@bgsm.edu

Participants

Suzy V. Torti, PhD
Assistant Professor, Biochemistry
Wake Forest University School of Medicine
Medical Center Boulevard
Winston-Salem, NC 27157
336-716-9357
336-716-0293
storrti@bgsm.edu

Fernando Tricta, MD
Medical Advisor
Apotex Research
150 Signet Drive
Toronto, Canada M9L 1T9
416-401-7332
416-741-0412
ftricta@apotex.ca

Chris Vulpe, MD, PhD
Postdoctoral Fellow
University of California, San Francisco
2614 47th Avenue
San Francisco, CA 94116
415-502-5138
415-502-0720
vulpe@cgl.ucsf.edu

Janet Wiegand
Senior Biological Scientist
Department of Medicinal Chemistry
University of Florida
Room P-401
1600 S.W. Archer Road, P.O. Box 100485
Gainesville, FL 32610
352-846-1956
352-392-8406

An-Sheng Zhang, MD
Research Associate
Lady Davis Institute of Jewish General Hospital
3755 Chemin Cote St.-Catherine Road
Montreal, Canada P.Q. H3T 1E2
514-340-8260 x 4666
514-340-7502
cxzg@musica.mcgill.ca

Kinetics and Mechanism of the Fenton Reaction. Implications for Iron Toxicity.

Willem H. Koppenol, PhD

Laboratorium für Anorganische Chemie, Eidgenössische Technische Hochschule, Zürich, Switzerland

Iron can play a role in the formation of oxyradicals: (i) autoxidation yields $O_2^{\bullet -}$, and (ii) the one-electron reduction of peroxides yield $HO^{\bullet}$ or $RO^{\bullet}$. The reaction of Fe(II)L, in which L is the ligand, with H_2O_2, the Fenton reaction, may also form an iron(IV)oxo complex. The reduction potentials of the couples $O_2/O_2^{\bullet -}$ and H_2O_2, $H^+/HO^{\bullet}$, H2O are (0.16 V and +0.32 V at pH 7 and 1 molal concentrations, respectively. Thus, a metal complex with a reduction potential of (0.16 V can reduce oxygen to superoxide, but a somewhat higher potential will still suffice as superoxide rapidly disappears. The same holds even more so for the one-electron reduction of peroxides. Kinetically, the reduction of dioxygen and peroxides appear to be inner-sphere redox reactions. Iron sequestered in proteins does not participate in the reduction of either dioxygen or peroxides, although the form-ation of superoxide in the respiratory chain is appreciable.

The concentration of redox-active iron *in vivo* is not known with certainty, but is likely to be in the micromolar range under normal conditions. The biological ligands are also unknown, and therefore the rate constants are unknown. Experiments with citrate, adp and atp give rate constants of the Fenton reaction of a few thousand per mole and per second, and activation energies of ca. 40 kJ/mol. Rate constants of organic peroxides are smaller by a factor of two.[1]

Iron can be made redox-inactive by forming complexes that either have very high or very low reduction potentials. An example of the latter is the ferrioxamine complex. Similarly, a Fe(II) $(phenanthroline)_3$ complex requires an oxidizing agent with a reduction potential in excess of 1 V. Kinetically, iron(II)complexes are somewhat labile, so that monodentate ligands never will prevent oxidation by either dioxygen or a peroxide. A polydentate ligand is necessary to make iron inert.

[1] W. H. Koppenol, Chemistry of iron and copper in radical reactions, in: *Free Radical Damage and its Control*, (C. A. Rice-Evans and R. H. Burdon, eds) Amsterdam, Elsevier Science B.V., 1994, p. 3-24.

Iron-Driven Damage to HbSS and Thalassemic Erythrocytes

Robert P. Hebbel, MD

University of Minnesota

Detailed studies of the sickle red blood cell have elucidated the biochemical components of oxidative damage to the red cell membrane. The autoxidation scenario includes several components, as follows. Sickle hemoglobin is modestly unstable, which results in generation of increased amounts of superoxide, peroxide and to hydroxyl radical. Sickle red cells generate each of these oxidant species to excess. Sickle red cells exhibit decompartmentalization of iron such that abnormal deposits of several iron types are found at the cytoplasm/membrane interface: free iron, denatured hemoglobin, and molecular iron. These pathologic membrane iron deposits are able to valance cycle, as driven by ascorbate or superoxide or lipid hydroperoxides. As a result of this oxidative chemistry taking place at the cytosol/membrane interface, the sickle membrane acquires abnormal amounts of lipid peroxidation byproducts and thiol oxidation. Many membrane defects can potentially be ascribed to this oxidative chemistry, but several stand out as being strongly supportable by data in the literature. (1) The oxidative denaturation of hemoglobin to hemichrome causes band 3 clumping, attraction of immunoglobulin, and excessive erythrophagocytosis. (2) Thiol oxidation abnormally stimulates the K:Cl co-transport pathway, leading to cellular dehydration. (3) Thiol oxidation is also implicated in the abnormal stiffness of the red cell membrane, contributing to abnormal red cell flow behavior. (4) Thiol oxidation probably underlies the abnormal vesiculation tendency of sickle red cells, providing procoagulant material to the blood. (5) Abnormal accumulation of lipid hydroperoxides confers an abnormal susceptibility to deformation-induced potassium leak, leading to dehydration. Notably, thalassemic red cells are very similar (and in many cases identical) to sickle red cells in the above respects. Consequently, to obtain the first experimental documentation of the role of pathologic iron in the red cell pathobiology of hemoglobinopathies, we administered a free iron chelator (L1) to thalassemic mice. As a result of this therapy, their pathologic deposits of red cell membrane molecular iron diminished significantly, and this was accompanied by improvements in cellular cation content, thiol oxidation, and red cell survival. Therefore, membrane-penetrating iron chelators may have the added therapeutic advantage of removing pathologic deposits of iron from the red cell cytoplasm/membrane interface, thereby ameliorating those aspects of red cell pathobiology that result from oxidative chemistry driven by membrane-associated iron.

Intracellular Iron and Cell Death

Ulf T. Brunk, MD, PhD

Department of Pathology II, University of Linköping, Sweden

Mammalian cells normally contain small amounts of redox-active iron within their lysosomes. This is a function of their continuously ongoing autophagocytotic degradation of various metallo-proteins. Since redox-active iron catalyzes the homolytic cleavage of hydrogen peroxide, under the formation of the very reactive hydroxyl radical, the influx of hydrogen peroxide into the lysosomal compartment is potentially risky. Oxidative stress, increased amounts of lysosomal low molecular weight iron, and decreased cellular capacity to degrade intra- or extracellularly formed hydrogen peroxide may lead to oxidative damage of lysosomal membranes, with ensuing leakage to the cytosol of lytic enzymes.

Limited such leak seems to induce transient autophagocytosis of reparative character, while moderate lysosomal rupture is followed by apoptosis within initially intact plasma membranes, probably by activation of the caspase cascade. In contrast, severe oxidative stress, which results in a more complete lysosomal breach, is associated with necrosis and cell lysis. Limiting the availability of intralysosomal low molecular iron in a redox-active form could therefore represent potential protection for cells under oxidative stress. Such protection may be accomplished by stimulating the endocytotic uptake of exogenous iron-chelators, such as desferrioxamine, or the endogenous apo-ferritin-synthesis with resultant lysosomal enrichment with ferritin via autophagocytosis. Such limitation of the redox-availability of intralysosomal iron largely prevents not only oxidative stress-induced lysosomal labilization, but apoptosis as well.

Cells with low capacity to degrade hydrogen peroxide, such as differentiated insulinoma cells, seems to be able to induce ferritin synthesis more rapidly than cells which easily degrade hydrogen peroxide. Such induction may be a defense mechanism against oxidative stress. Nevertheless, we hypothesize that ß-cells which, like insulinoma cells, have a weak anti-oxidative defense system under conditions of enhanced general autophagocytosis, or crinophagy, might become lethally vulnerable to even low, or moderate, oxidative stress of the magnitude that may be expected to occur in pancreatic islets within the microenvironment surrounding activated macrophages under oxidative burst (e.g., during autoimmune insulitis).

Damage to macrophages induced by oxidized low density lipoprotein (oxLDL) is associated with iron-mediated intralysosomal oxidative reactions, which cause partial lysosomal rupture and ensuing apoptosis. This series of events can be prevented by pre-exposing cells to desferrioxamine, whereas it is augmented by treatment of the cells with a low-molecular-weight iron-complex. This suggests that the normal contents of lysosomal redox-active iron may play an important role in oxLDL-induced cell damage, presumably by catalyzing intralysosomal fragmentation of lipid peroxides and the formation of toxic aldehydes and oxygen-centered radicals.

Since lipofuscin contains a considerable amount of iron, it is conceivable that the loading of the lysosomal compartment with this age-pigment within aged post-mitotic cells, such as nerve- and heart muscle cells, might render them abnormally sensitive to oxidative stress, and perhaps explain the common occurrence of nerve cell degeneration following TIA-attacks. The necrosis of hepatocytes and ß-cells in advanced heamochromatosis, may equally be explained by lysosomal overloading with redox-active iron, making these organelles increasingly sensitive to oxidative stress.

Iron Chelators, NF-κB Activation and Liver Disease

Hidekazu Tsukamoto, PhD

University of Southern California

NF-κB, a redox-sensitive transcription factor, transactivates the promoters of numerous genes involved in inflammatory and immune responses such as cytokines (e.g. TNFα, IL-6, IL-8), chemokines (MCP-1, MIP-1 and 2), growth factors (M-CSF), adhesion molecules (ICAM, VCAM) as well as oxidant and antioxidant enzymes (iNOS, SOD, GCS). Our research to date demonstrates that activation of NF-κB and induction of NF-κB responsive genes in hepatic macrophages (HM) are dependent on an intracellular, chelatable pool of iron and that sufficient chelation of this pool abrogates these molecular events.

In animal models of cholestatic and alcoholic liver injury, HM participates as critical effector cells to promote cytotoxic, proinflammatory or fibrogenic responses in the liver. These responses are in large mediated by activation of NF-κB and induction of the aforementioned mediators. The NF-κB mediated events by HM are shown to be prevented by *in vivo* or *ex vivo* treatment with a lipophilic chelator (1,2-dimethyl-3-hydroxypyrid-4-one, deferiprone). These effects are associated with normalization of the increased non-heme iron content in HM and amelioration of liver injury in the models, suggesting the pivotal role of iron in HM NF-κB activation and subsequent pathogenetic processes.

The increased HM non-heme iron content in the alcoholic liver injury is accompanied by 2-fold increases in ferritin protein and L-chain mRNA expression in HM as well as in the splenic non-heme iron content. Further, hemeoxygenase-I mRNA expression is upregulated in HM, suggesting enhanced heme turnover as a mechanism of the increased iron storage. Recapitulation of this hypothetical mechanism in vitro by phagocytosis of heat-treated erythrocytes, increases the cellular iron content and accentuates subsequent LPS-stimulated NF-κB activation in cultured HM. Addition of ironic iron (~50μM) to cultured HM causes a transient decrease in the cytosolic IκBα and sustained increases in p65 and DNA binding of the p65/p50 heterodimer and the p50/p50 homodimer in nuclear extracts, demonstrating direct activation of NF-κB by iron. DNA binding of AP-1 is not affected by the same treatment. Further, LPS-induced NF-κB activation is preceded by a transient increase in the chelatable pool of intracellular iron, and the pretreatment of the cells with deferiprone (100 μM), abrogates the increases in both iron and NF-κB binding. Interestingly, this inhibitory effect of deperiprone on NF-κB appears dose-dependent since lower concentrations of the chelator (<1 μM) actually increase NF-κB binding in HM.

In summary, these results suggest: 1) the causal role of chelatable intracellular iron in NF-κB activation in HM both in vitro and in vivo; 2) increased HM iron storage as a priming mechanism for NF-κB activation and cytokine expression; and 3) dose-dependent biphasic effects of deferiprone on HM NF-κB activation. (Supported by USPHS grants R37 AA06603 and P30 DK48522, and Medical Research Service of Department of Veterans Affairs).

Iron, DNA and Chromatin Remodeling

Sally A. Leong,[1,2] W.M. Yuan,[2] and L. Kearney.[2]

USDA, ARS Plant Disease Resistance Research Unit,[1] Department of Plant Pathology,[2]
University of Wisconsin

Iron present in loosely bound or unchelated forms within cells can act catalytically to produce DNA damaging and lipid peroxidizing oxygen radicals. Living cells regulate the transport and storage of iron to minimize free radical damage. When this regulation is disrupted, the consequences on cellular growth and survival are dramatic. Mutations in *fur*, a gene regulating high affinity iron uptake in *Escherichia coli*, lead to 8-fold elevated levels of intracellular iron (Keyer and Imlay, 1996) and increased rates of cell death and mutagenesis during aerobic growth (Keyer and Imlay, 1996; Touati *et al.*, 1995). Likewise, an AFT1^{up} mutation in yeast leads to deregulated iron uptake and reduced cell growth in iron-replete medium (Yamaguchi-Iwai *et al.*, 1995). In both systems, the effects of iron overload are aggravated by mutations in DNA repair systems demonstrating that DNA damage is prevalent under these conditions (Touati et al., 1995; Philpott et al., 1996). In humans, hemachromatosis leads to iron loading in the liver, heart and pancreas, resulting in cirrhosis of the liver, pancreatic fibrosis and cardiac dysfunction, as well as increased risk of hepatocellular carcinoma (Crawford *et al.*, 1996). Moreover, an iron-unresponsive mutant of the IRP protein, which normally controls iron uptake and storage at the cellular level, results in diminished cell survival (DeRusso et al., 1995). Despite the wealth of information on the negative impact of iron overload in cells, surprising little is known about the molecular basis of iron-mediated, transcriptional control of iron uptake in eucaryotes where transcription occurs on a chromatin template.

In response to iron starvation, the basidiomycete fungus *Ustilago maydis* produces two cyclic hydroxamate siderophores ferrichrome and ferrichrome A. Three genes required for siderophore biosynthesis and regulation have been characterized: *sid1* encodes ornithine-N^5-oxygenase, the first enzyme in the ferrichrome biosynthetic pathway; *sid2* encodes a putative peptide synthetase required for ferrichrome biosynthesis; and *urbs1* encodes a transcription repressor that interacts via its C-terminal finger domain with GATA sequences in the *sid1* (Leong and Winkelmann, 1998). Our working hypothesis is that iron modulates siderophore gene expression at one or more levels. Iron may act as a corepressor of genes regulated by Urbs1 and as a physiological effector that indirectly affects expression of siderophore genes by altering the structure and/or cellular location of Urbs1. As with Fur, the procaryotic analog of Urbs1, iron may directly activate Urbs1 in DNA binding. Urbs1 may repress expression of *sid1* through positioning of nucleosomes and/or by formation of a DNA loop in the *sid1* upstream region. Consistent with this latter hypothesis, discrete and iron-dependent, nuclease hypersensitivity sites have been identified near the origin of transcription initiation of *sid1*.

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Mechanism of Hemopexin-Mediated Protection Against Oxidative Reactions Due to Elevated Extracellular Heme

Ann Smith, PhD

Division of Molecular Biology & Biochemistry, School of Biological Sciences, University of Missouri–K.C.

The plasma glycoprotein hemopexin is the primary physiological transporter of heme and is in the first line of defense against deleterious effects of heme. By binding heme, hemopexin abrogates its oxidative effects which are considered to contribute to the pathology of hemolytic conditions, reperfusion after ischemia, neurodegenerative diseases, stroke and aging. High (2-20 μM) but not low (0.01-1 μM) concentrations of heme-hemopexin increase the cellular protein carbonyl content and the DNA binding of transcription factors known to respond to oxidative stress, including NFκB. Heme-hemopexin is a rapid activator of the N-terminal c-Jun kinase/stress activated protein kinase (JNK/SAPK), and free heme is without effect on this kinase in spite of increasing protein carbonyl production to similar levels. The heme analog cobalt-protoporphyrin IX (CoPP) bound to hemopexin is an avid ligand of the hemopexin receptor but no tetrapyrrole uptake occurs. This complex allows the role of activation of signaling pathways by receptor occupancy to be investigated independently of transport. CoPP-hemopexin does not increase the carbonyl content of cells but does activate JNK/SAPK. Increased intracellular heme levels taken up *via* the hemopexin receptor are neither toxic nor apoptotic but rather induce cell arrest, whereas nanomolar concentrations of heme-hemopexin stimulate growth in part by supplying nutrient iron. Cu(I) is generated at the cell surface by redox processes in an early event in the induction of MT-1 and HO-1 mRNA by heme-hemopexin. Several transcription factors, whose DNA binding is increased in cells exposed to oxidative stress are affected by the extracellular levels of heme-hemopexin. Bathocuproinedisulfonate (BCDS), which specifically chelates Cu(I), prevents the increased intracellular oxidation state in response to heme-hemopexin. The nuclear translocation of two transcription factors, NFκB and the metal-responsive element binding protein, MTF-1, also known to respond to oxidative stress, is due principally to events due to hemopexin receptor occupancy. The hemopexin receptor acts as a sensor of the extracellular environment since receptor occupancy activates signaling cascades for key transcription factors and uptake of heme *via* the hemopexin system allows controlled heme transport at rates which allow cells to respond and protect themselves not only from the transient increased oxidation state associated with heme transport and catabolism but also from subsequent exposures to heme. Since free heme does not induce JNK, the signaling events, like sustained phosphorylation of c-Jun, G2/M arrest and increased expression of the cell cycle inhibitor $p21^{WAF1/CIP1/SID1}$, generated by heme-hemopexin appear to be of paramount importance in cellular protection by hemopexin. Perfusion after ischemia induces nuclear levels of c-Jun and NFκB thus, heme-hemopexin is likely to be a key player in these cellular responses *in vivo*. The hemopexin receptor-mediated heme uptake system allows controlled heme transport at rates which allow cells to respond and protect themselves *via* concomitant gene regulation activated by occupancy of the surface receptor. Hemopexin protects cells lacking hemopexin receptors by limiting their exposure to heme and targeting it elsewhere.

Critical Steps in Transmembrane Iron Transport

Nancy C. Andrews MD, PhD

Howard Hughes Medical Institute and Division of Hematology/Oncology,
Children's Hospital and Department of Pediatrics, Harvard Medical School, Boston, MA

Three aspects of mammalian iron transport have been heavily investigated, particularly in recent years. Our laboratory has used animal models with defects in various aspects of iron absorption, transport and utilization to study these processes. We have mapped spontaneous mutations in rodents with inherited iron deficiency anemia, and we have generated new mutant mouse models by gene targeting.

Transmembrane iron transport: Until recently, it was not known how iron moves across biological membranes in mammals. Through genetic experiments, we found that natural resistance associated macrophage protein 2 (Nramp2, also known as DCT1) is important for iron transport. We used a positional cloning/candidate gene approach to show that the microcytosis (*mk*) strain of mutant mice and the Belgrade (*b*) strain of mutant rats both have the same missense mutation (G185R) in *Nramp2*. This mutation results in severe impairment of transmembrane iron transport, particularly affecting apical iron absorption in the small intestine, and endosomal iron transport in erythroid precursors. Combined with biochemical evidence from Gunshin, Hediger and co-workers, our results indicate that Nramp2 is the major transmembrane iron transporter in mammals. We have recently proposed that Nramp2 be renamed DMT1, for divalent metal transporter 1, to give a more accurate indication of its activity.

Transferrin cycle: Transferrin (Tf) acts as a plasma chelator of iron. Through interaction with specific Tf receptors (TfRs), diferric transferrin enters an endosomal compartment of the cell, and supplies a concentrated source of iron for uptake. We have disrupted the gene encoding mouse TfR in ES cells, and used the mutant cells to produce mice with TfR -/- and TfR +/- genotypes. TfR-/- mice die *in utero* at embryonic day 10-11. Many TfR-/- embryos have severe anemia, presumably because iron uptake by erythroid precursors cannot meet the demands of hemoglobin production. Other TfR-/- embryos do not appear anemic, but still die *in utero*, apparently as a result of increased cell death in the vicinity of the developing neural tube. Apart from these defects, TfR-/- embryos are well formed. Heterozygous TfR+/- animals are liveborn, and appear grossly normal. Surprisingly, however, they have hypochromic, microcytic red cells, associated with varying degrees of anemia. This suggests that haploinsufficiency for TfR is sufficiently deleterious to cause a red blood cell phenotype. This has implications for human patients with inherited anemias.

Hfe regulation: Hfe is the molecule affected in patients with hereditary hemochromatosis. We have made two types of mutations in the murine *Hfe* gene. We have disrupted the gene, to produce Hfe knockout animals, and we have introduced the C282Y mutation (present in human patients with hemochromatosis) into the murine gene to produce an animal model of human hemochromatosis. The difference in phenotypes observed for Hfe-/- versus HfeC282Y/C282Y animals is informative. Iron loading is more severe in animals with the knockout mutation than in animals with the missense mutation. This suggests that human hemochromatosis represents a relatively mild alteration of a gene that is very important in normal iron metabolism.

Analysis of Human Iron Disorders Through the Study of Transition Metal Metabolism in Yeast

Jerry Kaplan, PhD

University of Utah, School of Medicine, Salt Lake City, UT

While required by all prokaryotes and most eukaryotes, iron in excess can be toxic. Organisms have developed sophisticated mechanisms to tightly regulate the concentration of iron within cells and within organelles. Malregulation of iron transport and metabolism result in disease. Through the study of the model eukaryote, the budding yeast, many human diseases of iron or other transition metals can be analyzed on a genetic and biochemical level.

High affinity iron transport in both humans and yeast relies on a transport system that consists of a multicopper oxidase and a ferric transporter. Cellular iron accumulation in yeast requires the ferroxidase activity of the multicopper oxidase to convert ferrous to ferric iron, which is transported across the cell surface by the permease Ftr1p. In humans, ferrous iron in plasma is oxidized by ceruloplasmin, which loads iron on to transferrin. Iron is delivered to the erythron and rapidly dividing cells, cells that have the highest iron requirements. Defects in the assembly of the yeast or human ferroxidase lead to decreased iron transport in yeast, and disease in humans due to an excessive deposition of iron or copper in tissues.

Human disease results not only from malregulation of cellular iron accumulation, but from alterations in intracellular iron metabolism. A number of intracellular transporters deliver iron to specific organelles. Defects in these transporters affect the movement of iron resulting in an iron maldistribution within cells. This maldistribution may result in organelle excess and cytosolic deficiency. Mutations in the yeast YFH1 gene, a homologue to the human gene frataxin, results in mitochondrial iron excess and respiratory deficit. Defects in the human frataxin gene result in Freidreichs' Ataxia, a lethal neuralogical and cardiac disorder. Both the human and yeast genes are nuclear genes that encode a mitochondrial protein. The yeast gene is required for proper mitochondrial iron export, and when defective results in mitochondrial iron accumulation. These results provide a model for the pathophysiology of Freidriechs' Ataxia suggest that it is a disease or organelle iron overload.

Metabolism and Transport of Iron: Distinct Control Mechanisms in Erythroid Cells

Premsyl Ponka, MD, PhD

Departments of Physiology and Medicine, McGill University and Lady Davis Institute, Jewish General Hospital, Montreal, Canada.

All cells in the organism require iron (Fe) and, in general, control its uptake and storage by a mechanism moderated by interplays between iron-responsive elements (IRE) and iron-regulatory proteins (IRP). However, many specialized cells and tissues have specific requirements for Fe and appear to display distinct regulation of its uptake and, perhaps, intracellular trafficking. In at least some instances, cells evolved mechanisms that can override the ubiquitous IRE/IRP control system. Distinct Fe metabolism is best exemplified by erythroid cells which are the most avid consumers of Fe in the organism. Virtually all the Fe taken up by immature erythroid cells appears in heme whose rate of synthesis (on a per cell basis) is at least one order of magnitude higher than that in the liver. Differences in Fe metabolism and in genes for 5-aminolevulinic acid (ALA) synthase are responsible for the differences in regulation and rates of heme synthesis in erythroid and nonerythroid cells. In nonerythroid cells, the rate-limiting and controlling step of heme production is catalyzed by ALA synthase (ALA-S). However, since the 5' untranslated region of the erythroid-specific ALA-S mRNA contains the IRE, a *cis*-acting sequence responsible for translational induction of erythroid ALA-S by Fe, the availability of Fe controls protoporphyrin IX levels in hemoglobin-synthesizing cells. Erythroid cells obtain Fe exclusively from transferrin (Tf) *via* Tf receptors (TfR) that are transcriptionally "over-expressed". Our results indicate that TfR induction during erythroid differentiation is probably independent of the IRE/IRP regulatory system. Moreover, we have provided evidence that heme is essential for maintaining a normal rate of TfR synthesis in erythroid cells. While succinylacetone (SA, an inhibitor of ALA dehydratase) inhibits TfR expression in differentiated murine erythroleukemia (MEL) cells, 48h-incubation of MEL cells with ALA (0.06-1 mM) results in a dose-dependent increase in TfR mRNA levels that is accompanied by an enhanced uptake of ^{125}I-Tf by the cells. ALA-mediated enhancement of TfR mRNA could be prevented by SA, indicating that the effect required the conversion of ALA into heme. Interestingly, control and ALA-treated MEL cells contained identical levels of active IRP-1, suggesting that endogenous heme may stimulate TfR expression by a transcriptional mechanism. In erythroid cells Fe acquired from Tf is specifically targeted to mitochondria where ferrochelatase inserts the Fe into protoporphyrin IX to form heme. This is documented by our results showing that in hemoglobin-synthesizing cells Fe acquired from Tf continues to flow into mitochondria, even when the synthesis of protoporphyrin IX is markedly suppressed. In addition, we have found that the inhibition of endosome motility decreases the rate of ^{59}Fe incorporation from ^{59}Fe-labelled endosomes into heme. These observations, together with our recent confocal microscopy studies, suggest that in erythroid cells a transient mitochondria-endosome interaction may be involved in Fe translocation to ferrochelatase.

Regulation of Erythropoiesis

H. Franklin Bunn, MD

Harvard Medical School, Boston, MA

The physiologic regulation of the red cell mass depends upon enhanced transcription of the erythropoietin (Epo) gene in response to hypoxia. Studies of Epo gene expression have been useful in investigating the mechanism by which cells and tissues sense hypoxia and respond with biologically appropriate alterations in gene expression. Both *in vivo* and in cell culture, the expression of Epo and other genes of physiologic relevance such as vascular endothelial growth factor and glycolytic enzymes is induced not only by hypoxia but also by certain transition metals (cobalt and nickel) and by iron chelation. It is likely that oxygen sensing involves a heme protein in which cobalt and nickel can substitute for iron in the porphyrin ring. Indirect evidence suggests that the sensor is present in all cells, and is a multi-subunit assembly containing an NAD(P)H oxidase capable of generating peroxide and reactive oxygen intermediates which serve as signaling molecules.

The up-regulation of Epo gene transcription by hypoxia depends on at least two known DNA binding transcription factors, HIF-1 and HNF-4, which bind to cognate response elements in a critical ~50 bp 3' enhancer. HIF-1 binding is induced by hypoxia as well as by cobalt and iron chelation. HIF-1 is a bHLH heterodimer which is activated in a wide range of tissues challenged by hypoxia and mediates the regulation of a number of O_2 responsive genes. HIF-1α and HIF-1ß mRNAs are constitutively expressed and not significantly affected by changes in oxygen tension. The activation of HIF-1 by hypoxia depends upon the selective protection of its α subunit from ubiquitin-dependent proteolysis, by means of a mechanism that depends on both phosphorylation and redox chemistry. We have recently identified a central domain in HIF-1α which is both necessary and sufficient for oxygen-dependent degradation.

HNF-4 is an orphan nuclear receptor which is constitutively expressed in kidney and liver, and cooperates with HIF-1 to give maximal hypoxic induction. The C-terminal activation domain of HNF-4 binds specifically to the ß subunit of HIF-1 while the C-terminal portion of the α subunit of HIF-1 binds specifically to p300, a general transcriptional activator. This very large protein and its homologue CREB binding protein (CBP), are inactivated by binding to the adenovirus protein E1A. Hypoxic induction of the endogenous Epo gene in Hep3B cells as well as an Epo-reporter gene is fully inhibited by E1A but only slightly by a mutant E1A that fails to bind to p300. Moreover, over-expression of p300 enhanced hypoxic induction. Thus, in hypoxic cells, p300 or a related family member forms a macromolecular assembly with HIF-1 and HNF-4, enabling transduction from the Epo 3' enhancer to the apparatus on the promoter responsible for the initiation of transcription.

The Role of Iron Regulatory Proteins in Maintenance of Iron Homeostasis in Mammals

Tracey Rouault, MD

National Institute of Child Health and Human Development, NIH

Iron regulatory proteins (IRPs) are proteins that sense cytosolic iron levels and accordingly modify expression of iron metabolism proteins. The two mammalian IRPs are similar to one another, but the mechanism for iron sensing differs between the two proteins. IRP1 is an iron-sulfur protein that functions either as a cytosolic aconitase or as an RNA binding protein, depending on cellular iron status. Insertion of an iron-sulfur cluster into the active site cleft of IRP1 results in loss of binding to RNA stem-loops known as iron regulatory elements (IREs). These stem-loops are found in the transcripts of numerous iron metabolism proteins, including ferritin, transferrin receptor (TfR), erythrocyte ALA synthase, mammalian mitochondrial aconitase, and Drosophila succinate dehydrogenase. An IRE is also present in the 3'UTR of DCT1/ Nramp2, the recently cloned iron transporter responsible for microcytic anemia in mice. Consensus IREs are binding sites for both IRPs; binding of IRPs to IREs near the mRNA cap site results in decreased translation, whereas binding to IREs within the 3'UTR stabilizes the mRNA of TfR, and possibly DCT1.

IRP2 is degraded in iron-replete cells by a pathway that involves iron-dependent oxidation of the protein, ubiquitination, and degradation by the proteasome. Thus, IRE binding activity of both IRP1 and IRP2 is decreased in iron-replete cells. Both IRPs are found in cells, and the activities are theoretically redundant. However, targeted disruption of IRP2 in mice results in an increase in intestinal iron uptake and a progressive neurologic syndrome. Possible explanations for the phenotype of the IRP2-/- mice will be discussed.

Modulation of Ferritin Expression and Iron Metabolism by Ascorbic Acid and by Hypoxia

Ildiko Toth, Hemant Thatte, Liping Yuan, Jack T. Rogers, Hayden Boyce, David Golan, Kenneth R. Bridges

Brigham and Women's Hospital, Harvard Medical School, Boston, MA

A key component of the regulation of ferritin expression involves a post-transcriptional mechanism in which the iron response protein (IRP) binds to the ferritin message at IRE consensus recognition sequence. We have examined the effects of cell environmental factors on this process. In particular, we have examined the influence of ascorbic acid and of hypoxia on this process. Physiological concentration of ascorbic acid increase by four-fold the degree of *in vivo* phosphorylation of IRP-1 in K562 erythroleukemia cells. We found that ascorbic acid increases by two-fold the degree of *in vitro* phosphorylation of IRP-1 by protein kinase C when added to in vitro incubation mixes. Electromobility shift assay shows enhanced IRP/IRE binding in cells treated with ascorbic acid. The functional consequence of enhanced IRP/IRE binding was seen as an increase in transferrin receptor expression in the cell. This consistent with the enhanced transferrin receptor mRNA stability produced by IRP/IRE binding. Iron overload depletes the body supply of ascorbic acid. Strategies designed to chelate excess iron should factor the effects of modifed ascorbate levels in these patients.

Hypoxia, likewise, alters IRP/IRE interaction. Using the human hepatoma cell line Hep3B as a model, we found that a 16 hour exposure to a 1% oxygen atmosphere markedly increases IRP/IRE binding as assessed by electromobility shift assay. Hypoxia also decreases cytosolic aconitase activity in Hep3B and in K562 human erythroleukemia cells. In the case of the transferrin receptor, the hypoxia-enhanced IRP/IRE binding stabilized the message and increased the cellular mRNA content by over 10-fold. The expression of the transferrin receptor doubled in these cell during 16 hours of hypoxia. Simultaneously, enhanced IRP/IRE binding suppressed translation of the ferritin message. The effect of hypoxia was most strikingly depicted by the absence of ferritin synthesis in cells challenged with inorganic iron. The suppression of ferritin synthesis means that hypoxic cells are susceptible to iron-mediated oxidant damage once the oxygen tension returns to normal. This sequence of events could contribute to the cell and tissue damage in patients with iron overload and concurrent regional hypoxia, as may occur in sickle cell disease, for instance.

A New Twist in Ferritin Iron Release

E. C. Theil and H. Takagi

Children's Hospital Oakland Research Institute, Oakland, CA

Ferritin iron uptake and iron release are each regulated in cells. Structure/function of both the genetic regulatory elements and the protein are so highly conserved that model systems are unusually accurate analogues of human cells. Thus studies in humans, other mammals, birds, amphibia and prokaryotes are essentially superimposable. Understanding mechanisms that control iron release from ferritin will have important consequences for developing novel strategies to manage iron overload.

Iron uptake rates are regulated by altering the ratio of mRNAs for H and L type ferritin subunits: ferritin composed of H-type subunits takes up iron ~100-10,000 times as fast as ferritin composed of L-subunits. Tissue-specific iron uptake rates are achieved through genetically controlled variations in the ratios of H and L-type subunits. Ferritin synthesis rates are regulated by specific interactions between an mRNA regulatory element (IRE) and two regulator proteins (IRP1 and IRP2). Integration of ferritin synthesis with the synthesis of ferritin of other proteins of iron and oxidative metabolism, such as the transferrin receptor, erythroid aminolevulinate synthase, and m-aconitase, is achieved by differential IRP interactions with isoforms of theferritin IRE in the other mRNAs.

Iron release has been less studied than iron uptake because *in vitro*, the similarity of rates is so small that no hints about mechanisms could be obtained. In contrast iron release rates *in vivo* vary considerably among ferritins in different tissues suggesting that cytoplasmic components may interact with ferritin to modulate iron release. Recently a clue to a possible cytoplasmic mechanism for control of iron release was obtained with recombinant ferritins (Takagi et al.(J. Biol. Chem 273, 18685-18688,1998). A single amino acid substitution L134P, at a highly conserved site,increased the rate of reductive iron release 5x in a ferritins, whereas ron release from recombinant ferritin (H or L-subunit type) varied little. Complete dissolution of a 480 Fe atom mineral took 30x longer in the wild type the altered ferritin; none of the iron was released from either the wild type or the altered ferritin until the reductant (FMN/NADH) was added. X-ray crystallography indicated that the effect of the mutation was amplified because L134 is near the junction of three subunits and created a disordered or untwisted site at each of the three subunit junction around the molecule. The reductant thus had better access to the mineral. *In vivo* a cytoplasmic factor might untwist ferritin in response to cell signals for iron. Manipulation of such untwisting, coupled to intracellular chelators could greatly improve therapy for iron overload.

Current Status of Chelation Therapy

C. Hershko, MD

Hebrew University, School of Medicine, Jerusalem, Israel

Although the iron chelating drug deferoxamine (DFO) has been available for clinical use for almost four decades, it has only gained acceptance as a useful therapeutic agent in the late seventies following the demonstration of its ability to deplete iron stores in thalassemic patients. In this overview I shall discuss briefly the pathophysiology of iron overload, the impact of long-term treatment with DFO and L1 on survival in thalassemia and then, conclude with the potential use of iron chelators in conditions unrelated to iron-overload. In thalassemia, iron overload is the product of multiple transfusions and increased iron absorption. Ineffective erythropoiesis and the associated vast expansion of the erythroid marrow result in a 10 to 15-fold increase in plasma iron turnover, with an outpouring of catabolic RBC iron exceeding the iron-carrying capacity of transferrin. This results in the emergence of non-transferrin-bound plasma iron (NTBI). The rate of NTBI deposition in myocardial and hepatic cells is over 200-fold faster than of transferrin iron and believed to be directly responsible for iron toxicity in thalassemia. In heart cell cultures NTBI induces peroxidative damage to the sarcolemma and the mitochondrial inner membrane, resulting in abnormal heart cell contractility and rhythmicity, reversible by DFO treatment. Clinical studies have shown that NTBI is directly available for iron chelator-binding and hence a major target of iron chelating therapy. Long-term DFO treatment has improved the life-expectancy of thalassemic patients, mainly by prevention of cardiac mortality. Likewise, symptomatic siderotic myocardiopathy is reversible by aggressive DFO treatment. The introduction of deferiprone (L1) has generated considerable interest as an orally effective alternative to DFO. The present controversy about the clinical usefulness of L1 is centered around the prevalence and severity of its side-effects; its ability to maintain a negative iron balance, and; its possible hepatotoxicity. Other synthetic iron chelators are presently evaluated for clinical use and will be discussed by other speakers at this symposium. An important aspect of iron chelating treatment is preventing harmful reactions unrelated to iron overload. The most well defined of these are *reperfusion injury; anthracycline toxicity* and; *intracellular pathogens*. Because iron deficiency may interfere with the normal immune response and aggravate, rather than inhibit infection, infection may be controlled by selective iron depletion in compartments essential for microbial growth. This is analogous with the redistribution of iron elicited by the inflammatory response. The antimicrobial effect of cytokines, mediated by intracellular iron depletion, can be mimicked by a strong iron chelator such as DFO. Intriguing new observations on the antimalarial effects of DFO and other iron chelators lend new meaning to the term "Nutritional Immunity" and open new channels for exploring the possibility of controlling infection by selective intracellular iron deprivation. Packaging the chelator in liposomes or red cell ghosts, or manipulating their lipid solubility to improve their delivery to appropriate target organs such as the macrophage system may greatly improve their efficiency. With the introduction of new, orally effective chelators, it is reasonable to expect that future research may lead to the development of novel strategies of diasease control by selective iron depletion.

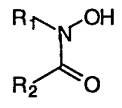
Introduction to Chelator Design

Kenneth Raymond, PhD

University of California—Berkeley

Chemists have for some time been rather good at making complexing agents for metal ions that have high affinities and so reduce the free aqueous metal ion concentration substantially. However such ligands, an example being EDTA, tend to be relatively nonselective. In contrast, nature provides examples of complexing agents that demonstrate not just high affinity for metal ions but also extraordinary selectivity. Siderophores, microbial ion transport agents, have high affinity and selectivity for ferric ion and have therefore been prototypes or models for therapeutic iron chelating agents. Some functional groups commonly found in siderophores, or associated with similar ligands, are shown below. Ferrioxamine B is the siderophore in the pharmaceutical Desferal®, which has for several decades been the therapeutic iron chelating agent in clinical use.

In addition to ease of administration and low toxicity, desirable chemical features of a chelator require a thermodynamic stability high enough to overcome *in vivo* complexation. Some examples will be provided of comparing such stabilities and their implications for iron chelation therapy. The kinetic ability of a chelating agent to remove iron from serum transferrin is also a significant factor and examples of this will also be presented, with specific reference to possible therapeutic applications and ligand design



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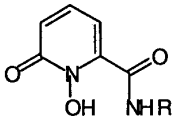

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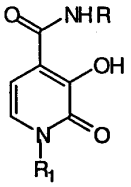

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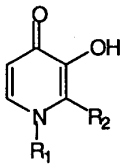

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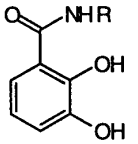
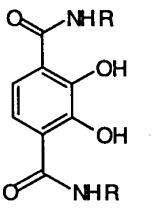


Catecholamide



2,3-Dihydroxy Terephthalamide

Clinical Toxicity of Iron Chelators

Beatrice E. Gee, MD

Morehouse School of Medicine, Atlanta, GA

Deferoxamine mesylate (DF) has been in use for over thirty years and consistent parenteral administration has been shown to be effective in controlling transfusional iron overload. While a generally safe medication, longterm use or high dosages of DF can cause a number of complications affecting various organs, most frequently the auditory nerves, eyes, kidneys, and bones. DF use is also associated with an increased risk of infection with *Yersinia enterocolitica* and some fungi. Such organisms appear to have a growth advantage when provided with iron-chelator complexes and DF may have some effect on host defenses.

Acute hypersensitivity reactions are rare and occur most commonly when DF is infused intravenously in large doses or at rapid rates. Auditory toxicity can include tinnitus or sensorineural hearing loss. Ocular toxicity may present as blurry vision or difficulty in adjusting to darkness. Formal ophthalmologic testing may reveal decreased visual acuity, visual field defects, decreased electro-retinographic responses, abnormal color vision, elevated dark-adapted thresholds, and retinal pigmentation. Some recovery of ocular function can occur after cessation of DF therapy but may not be complete. Ocular changes are relatively rare in individuals treated with "standard" dosages of DF. DF may cause mild to moderate decreases in glomerular filtration rate of the kidney and large doses of DF has been associated with rare cases of renal failure. The effects of DF on renal function are generally reversible when DF is stopped. Chronic DF treatment may also be associated with hypercalciuria and/or nephrocalcinosis. The renal loss of calcium and possibly other minerals may contribute to metabolic bone disease and osteopenia. Early institution and high levels of DF compliance may be associated with abnormalities in metaphyseal growth plates and slowed linear growth. These bony radiographic changes are distinct from those caused by bone marrow expansion. Osteopenia or osteoporosis is seen in many adults with thalassemia major and may also be related to DF therapy. DF toxicity may be more severe in individuals with lower iron burdens or those who have underlying organ dysfunction, such as diabetic retinopathy. The mechanisms of organ toxicity are not well-defined and may be related to the chelation of iron or other minerals from tissues.

1,2-dimethyl-3-hydroxypyrid-4-one (Deferiprone or L1) is a more recently studied oral iron chelator. Unlike DF, L1 is lipid soluble and has lower iron-binding affinity. Commonly experienced side effects of L1 included neutropenia or agranulo-cytois, arthropathy, and liver enzyme abnormalities. A recent study (Cragg et al.) showed that *in vitro*, L1 potentiated oxidative DNA damage in iron-loaded liver cells, especially when L1 was in lower concentrations relative to iron.

Clinical Effectiveness of Chelating Therapy

Nancy F. Olivieri, MD

University of Toronto, Canada

As a result of deferoxamine treatment, the prognosis for patients in countries able to afford this therapy has greatly improved. Modern regimens of deferoxamine reduce or normalize hepatic iron overload, improve linear growth, preserve anterior pituitary function, and extend survival free of the complications of iron overload if adequate control of iron burden is maintained. Over the past decade, a quantitative approach to the management of iron overload has been developed, providing guidelines for the control of body iron burden on individual patients treated with chelating therapy. While in practice, the serum ferritin are commonly used to assess treatment effectiveness, reliance on this test alone may lead to errors in management in individual patients: changes in body iron account for little more than half of the variation on serum ferritin concentrations. By contrast, measurement of the hepatic storage iron concentration, which is highly correlated with total body storage iron, provides the most quantitative, specific and sensitive method for evaluating body iron burden. Determination of heptic iron through liver biopsy performed under ultrasound guidance is safe, and permits rational adjustment of regiments of iron-chelating therapy tailored to individual patients. Magnetic suscpetometry provides a direct measure of hepatic storage that is quantitatively equivalent to that determined by biopsy of liver tissue over a range of iron concentrations. By contrast, magnetic resonance imaging does not provide an accurate measurement of hepatic iron concentration of patients with severe iron loading, hepatic fibrosis, or both. Because the magnitude of the body iron burden is the principal determinant of clinical outcome, the primary goal of iron-chelating therapy is adequate control of body iron. Body iron burdens corresponding to hepatic iron concentrations exceeding 15 milligrams iron per gram liver dry weight greatly increase the risk of cardiac disease and early death. By contrast, in a proportion of heterzygotes for hereditary hemochromatosis, slightly elevated body iron burdens (about 3.2 to 7 milligrams iron per gram liver, dry weight) are associated with normal life expectancy; homozygotes who develop iron burdens greater than this are at increased risk of complications of iron overload. This suggests that a conservation goal for iron-chelating therapy is to maintain a body iron corresponding to hepatic storage iron concentrations of about 3.2 to 7 milligrams iron per gram liver, dry weight. The serum ferritin concetrations corresponding to this range are less clearly defined.

continued on next page

Clinical Effectiveness of Chelating Therapy

Nancy F. Olivieri, MD

University of Toronto, Canada

The difficulties associated with deferoxaminc have led to a search for therapeutic alternatives. Short-term therapy with oral deferiprone (L1) was observed to have a favorable short-term effect on body iron burden, in the one study in which serial direct determinations of hepatic iron were obtained. Two extened studies have subsequent suggested that, during long-term deferiprone therapy, hepatic iron may stabalize at, or increase to, concentrations associated with increased risk for cardiac disease and early death in up to one-half of patients. In addition to the previously recognized adverse effects of embryotoxicity, teratogenicity, neutropenia and agranulocytosis, long-term deferiprone treatment has recently been reported to be associated with progression of hepatic fibrosis; the estimated odds of progression are estimated to increase by 5.8-fold with each additional year of therapy. These findings parallel those in two animal species in which deferiprone, and an hydroxpyridone structurally similar to deferiprone, was shown to increase hepatic and cardiac iron loading, worsen hepatic fibrosis, and induce cardiac and musculoskeletal fibrosis. By contrast, deferoxamine treatment arrests the progression of hepatic fibrosis even when hepatic iron is tabalized, rather than reduced. Taken together, these data suggest that deferiprone does not adequately control body iron burden in a substantial proportion of patients, and may promote worsening of hepatic fibrosis, confirming cautions previously expressed about the long-term administration of this agent in iron-leaded patients. Long-term followup of the effectiveness of other models of administration of defeoxamine, to date reported only on preliminary analyses, are awaited with interest. These include deferoxamine attached to high molecular starch, administered in twice daily subcutaneous bouses, and in lipid vehicle permitting slow release.

Transfusional Approaches to the Management of Iron Overload

Alan R. Cohen, MD

Division of Hematology, Children's Hospital of Philadelphia

Transfusion therapy, the cornerstone of the treatment of thalassemia major and other hematologic diseases, dramatically improves the lives of affected patients. However, iron overload is an inevitable, life-threatening complication of long-term administration of red cells. Iron chelation therapy with deferoxamine removes excessive iron, but the need for parenteral administration of the chelator, drug-related side effects and high cost lead to poor compliance and numerous treatment failures. An alternative approach to removing transfusional iron with chelators is to prevent or slow iron accumulation by modifying the transfusion therapy. This approach is most readily applied to sickle cell disease in which the goal of long-term transfusion therapy is usually the replacement of hemoglobin S cells with hemoglobin A cells. In previous studies we have demonstrated that raising the target hemoglobin S level from 30% to 50% decreases red cell requirements by approximately 33%. Using exchange transfusion in conjunction with the higher target hemoglobin S level decreases transfusion requirements by a mean of 67%. The greatest reductions occur with partial exchange transfusion by erythrocytapheresis (PET-E). Using this method, most patients with sickle cell disease do not require chelation therapy during chronic transfusion therapy. Thalassemia major presents a different challenge since the primary goal of transfusion therapy is to raise the total hemoglobin level. Judicious selection of a target pre-transfusion hemoglobin level within the range of 9-11 g/dl may minimize blood requirements but hemoglobin levels below this range are associated with significant complications. Increasing the frequency of transfusion while reducing the amount of blood administered each visit may also reduce the total blood requirements but many patients find such a schedule inconvenient. Young red cell (neocyte) transfusions should theoretically prolong the survival of administered erythrocytes, thereby decreasing total blood requirements. However, in clinical studies, the reduction in blood requirements with neocytes prepared from single donor units was only 13-16%, and the preparation costs and donor exposures were significantly higher in transfusion regimens employing neocytes in comparison with conventional red cell units. Berdoukas has combined PET-E with neocyte enrichment in the management of patients with thalassemia, achieving a 29% reduction in the rate of red cell loading. In a pilot study, we have performed 70 PET-Es without neocyte enrichment in three patients with thalassemia major. After determining optimal operating characteristics for PET-E, we have reduced net red cell loading by a mean of 45%. Donor blood utilization increased 2.5-3.5 fold in comparison with simple transfusion. Additional studies indicate that the beneficial effect of PET-E on net red cell loading (and therefore on the rate of iron accumulation) is directly related to a circulating red cell population of younger average age throughout the period between transfusions. The use of PET-E in sickle cell disease and thalassemia, and the investigation of this or different transfusion regimens in other hematologic disorders, may reduce or in some cases eliminate transfusional iron loading and the need for chelation therapy.

Alternative Approaches to Chelator Development

Raymond J. Bergeron, PhD

University of Florida

In recent years it has become clear that manipulating iron levels in particular biological compartments can have a profound effect on certain disease processes. The course of disorders as diverse as Cooley's anemia, inflammatory bowel disease, malaria, and reperfusion injury has been shown to be dependent on iron. Whereas the common denominator in exploiting iron as a therapeutic target in these diseases may be that of reducing "available" iron, very different design strategies are necessary; the parameters set by the nature of the disease on the toxicity profile and modes of chelator administration vary significantly.

Although Cooley's anemia probably best exemplifies the hurdles which must be overcome in drug design, it also demonstrates that it is possible to prematurely abandon potentially valuable drugs which, while they do not fall within the pharmacological boundary conditions set for this disease, would be useful for other indications. Patients with Cooley's anemia require lifelong treatment with iron chelators; therefore, two issues are of paramount importance in drug design considerations—the efficiency with which the ligand removes iron and long-term toxicity. The former issue has a tremendous impact on how the drug can be administered and thus on patient compliance. Whereas inflammatory bowel disease is a chronic problem, episodes are often sporadic; the resulting treatment is focused on symptomatic periods. Malaria and reperfusion injury are quite different regarding toxicity profile considerations inasmuch as short-term exposure is foreseen. Thus, ligands which are unacceptable for the treatment of iron overload associated with Cooley's anemia because of their long-term chronic toxicity may be completely appropriate and useful in other therapeutic arenas.

In order to illustrate the difficulties and some of the solutions to the problems in the design, testing, and selection of suitable iron chelators for treatment of iron-related disorders, studies with both natural product siderophores and synthetic ligands will be described. Most of the presentation will focus on the design and testing of ligands for transfusional iron overload, but the application of the compounds to the treatment of inflammatory bowel disease will be briefly outlined. These will include considerations of toxicity profiles and dosage regimen requirements dictated by the particular disease state.

Iron Chelation: Rationale for Combination Therapy

R.W. Grady, V.A. Berdoukas and P.J. Giardina

New York Hospital-Cornell Medical Center, New York, USA
and the Prince of Wales Children's Hospital, Sydney, Australia

Parenteral administration of desferrioxamine (DFO) has been the mainstay of iron chelation therapy despite numerous problems associated with its use, not the least of which is highly variable compliance. It was hoped that an orally effective chelator would obviate most of these problems, especially the latter. More than 20 years have now elapsed since the search for an oral chelator began in earnest. Thousands of compounds have been evaluated in various models of iron overload yet the ideal chelator has not materialized. Many of the most active compounds have proven to be too toxic, especially in normal animals. Compounds with a high affinity for iron will also bind other physiologically important metals such as Cu and Zn, albeit with lower affinities. Altering the homeostasis of these metals, binding the iron in essential pools and/or interacting with critical metalloenzymes is undoubtedly responsible for much of the toxicity observed. Moreover, the pathological pool of iron in various disease states is in different tissues/cellular compartments. Thus, both specificity and selectivity will determine whether a given chelator is suited to a particular situation. In some diseases, one may only need to inhibit a metalloenzyme to achieve the desired result while in others, like thalassemia, it is necessary to mobilize excess storage iron and cause its excretion. Furthermore, the best regimen to unload excess body iron stores may not be appropriate for preventing iron deposition in young children or for maintaining iron levels in those whose stores have been normalized. In the case of thalassemia, we suggest that a combination of chelators will prove to be the best solution, in particular, a combination involving use of a bi- or tridentate ligand to "shuttle" iron from storage sites to a hexadentate "sink" capable of forming a metabolically inactive iron complex which can be readily excreted. We have demonstrated the validity of this hypothesis both in the hypertransfused rat model of iron overload and in human balance studies. Deferiprone, one of the most promising oral agents, proved to be only 57% as effective as DFO (range 24%-129%) when fecal excretion of iron was taken into account. Given the nature of the side effects associated with deferiprone, significantly increasing the dose to enhance efficacy seemed impractical. Thus, we studied the effect of giving the drugs in combination. Deferiprone (75 mg/kg divided t.i.d.) was given with breakfast, dinner and a bedtime snack, DFO (60 mg/kg) being infused subcutaneously during the night. In 5/6 patients studied, the effect was additive with a shift to urinary iron excretion. Synergy was observed in the sixth. Since the chelatable pools of iron are rapidly refilled and the drugs were not given simultaneously, the significance of this result has been somewhat overlooked. Infusing DFO during the day or asking a patient to get up during the night to take deferiprone is unrealistic. This regimen is a practical approach to markedly increasing iron excretion in a minimally intrusive way. If the chelators were given simultaneously, one might expect a synergistic effect. This is most practical in the case of oral chelators. In 2/2 patients, combining N,N'-bis(o-hydroxybenzyl) ethylenediamine-N,N'-diacetic acid (HBED) and deferiprone resulted in synergy, with a shift towards urinary excretion of iron, supporting the "shuttle" hypothesis. The challenge now is to develop a variety of chelation regimens to be used in different situations, thereby giving patients more options to choose from and improving overall compliance.

ICL670A: A Novel Orally Active Iron Chelator

Hans Peter Schnebli, PhD

Pharmaceuticals Research, Novartis Pharma, Basel, Switzerland

ICL670A is an N-substituted bis-hydroxyphenyl-triazole, a representative of a new class of tridentate iron chelators. Forty-four compounds of the triazole series with different physico-chemical properties (e.g. water solubility, lipophilicity profile, iron-binding affinity and global charge of their iron complex) were synthesized. These compounds were evaluated both for their pharmacological effects and tolerability, together with some 700 other compounds from five additional chemical classes. ICL670A emerged from this selection process as the compound which best combines high oral potency and tolerability.

The potent and specific ability of ICL670A to mobilize tissue iron and promote its excretion has been demonstrated in several models: in the bile duct cannulated rat, ICL670A has a rapid onset of action and is five-fold more potent than Desferal® and ten-fold more potent than L1. In a chronic (12-week) study in iron-overloaded rats, ICL670A was twice as effective as Desferal and 5 fold more effective than L1. In the iron-overloaded marmoset, the most relevant model for the characterization of iron chelators, ICL670A induced iron excretion within a few hours of administration. Considerable amounts of iron were excreted in the 24- to 48-hour period, particularly at higher doses. In the marmoset, at equivalent (molar) doses, ICL670A was more than 10-fold more effective than L1. ICL670A induced iron excretion is predominantly by the fecal route and only a small percentage was excreted in the urine. ICL670A is highly selective for iron, and did not induce the excretion of zinc or copper in marmosets. Toxicity has been assessed both in rats and marmosets. With NOAELs of 65 mg p.o./kg/day in males and 130 mg p.o./kg/day in females in the 4-week study, marmosets tolerated the compound well. The kidney (i.e. alterations in the proximal tubular epithelium of both rats and marmosets), the gastrointestinal tract and the heart (rats) were identified as primary target organs of toxicity in these studies. Most of the treatment-related effects can be regarded as a direct result of extreme iron deprivation, as these correlated well with measured decreases in liver and kidney iron concentrations in the animals. The conclusion, that iron deprivation is the most probable mechanism of toxicity is strengthened by the fact that a similar pattern of nephrotoxicity has been observed with potent, orally active iron chelators of other chemical classes. Studies performed with ICL670A in iron-overloaded animals and with kidney cells *in vitro* further support the conclusion that most of the observed toxic effects relate to the effective sequestration of non-heme tissue iron, which is not expected to occur under conditions of therapeutic use in patients with iron-overload diseases. ICL670A is Ames-negative. In the context of its safety evaluation, it was furthermore shown that ICL670A did not increase the absorption of dietary iron in rats.

Orally administered ICL670A was rapidly and well absorbed in rats, marmosets and dogs. A dispersible tablet was selected based on its pharmacokinetic properties in Beagle dogs. From this formulation ICL670A was rapidly absorbed and maximal plasma concentrations were reached in less than 1 hour; the absolute bioavailability was near 100%.

Novartis Chelators Program: Clinical Development Update

Daniele Alberti, MD

Novartis International Clinical Research, Basel, Switzerland

Desferal® is an established and effective medication for the treatment of iron overload. Originally Desferal® was administered by the intramuscular route. This has subsequently been replaced by the subcutaneous (s.c.) and intravenous routes which provide better iron chelation efficiency. The current standard treatment of chronic iron overload in thalassaemia is by chelation with Desferal®. It is usually administered as a slow s.c. infusion preferably over a period of 8-12 hours; 5-7 times a week. A novel salt of desferrioxamine has been developed with properties suitable for formulation as an injectable depot. The rationale supporting the depot injection is to deliver a smaller dose of desferrioxamine over a longer period of time making it more efficient and thus reducing the proportion of non chelated 'wasted' desferrioxamine which can be up to 90%, depending on the dose and degree of iron overload.

The objective of the development programme is to develop ICL749B as an injectable depot formulation of desferrioxamine which can be used as a s.c. bolus injection of clinically acceptable volume given once every one or two days. Children and adolescent patients will be eligible for all clinical studies in the program.

Data from preclinical studies in animal models with the depot formulation have shown that the duration of action (≥ 30 h) and the cumulative iron excretion was at least 3 times that of Desferal® given as a s.c. bolus injection. In patients, the pharmacokinetic and metabolic data echo the pharmacodynamic behaviour. Mean AUC for the depot was 6 times greater than that of s.c. bolus injection. Duration of exposure to desferrioxamine was increased with the depot formulation; mean half-life for the terminal elimination phase in plasma was estimated to be 36.3 h in patients nominally dosed with 7.5 mg/kg s.c. CGH 749B, and 7.59 h, in the same group of patients infused with 40 mg/kg Desferal® over 8 hours.

Some slight irritation has been observed at the site of injection in 2-w and 13-w animal toxicity studies. No relevant systemic finding were observed in these studies.

The clinical development of ICL749B has started in January 1997. The results of a Phase I single administration study showed urinary iron excretion of clinical relevance with all of the ICL749B doses evaluated. On average, between 5-12 mg/kg of ICL749B corresponded to 40 mg/kg Desferal® infusion, with an equivalence point of 6.7 mg/kg. The tolerability profile was acceptable. These results endorsed the implementation of three Ph IIa repeated administration studies in thalassemic patients.Subject to the demostration of a satisfactory local and systemic safety profile in the earlier studies, pivotal trials will start in 1 Q 1999.

Update on Depot Desferrioxamine (ICL749B); Implications for Chelation Regimens

J.B. Porter[1], D. Alberti,[2] I. Hassan,[3] A. Racine,[4] B.C. Davis[1], V. Voi[5], and A. Piga[5]

University College London, UK[1], Novartis Pharma Ltd., Origgio, Italy[2], Novartis Pharma Ltd., Horsham, UK[3], Novartis Pharma Ltd., Basel, Switzerland[4], University of Turin, Italy[5]

Single dose Phase I studies with a new depot formulation of desferrioxamine (CGH 749B), aimed at providing continuous chelation without the need of a pump, have been completed. The relationship found between the pharmacokinetics and pharmacodynamics in these studies, have been used to model the likely plasma levels of drug and metabolite with repeat dosing and to design the dosing regimens for Phase II studies. In the Phase I studies, the tolerability profile, pharmacokinetics and urine iron excretion (UIE) were compared over 72 h (19 patients) and 144h (10 patients) after the administration of single subcutaneous bolus injections of CGH 749B (five escalating doses of 2.5, 5.0, 7.5, 10.0 and 12.5 mg/kg). with that of standard Desferal® (DFO) treatment (40 mg/kg, single subcutaneous infusion lasting 8 h). 28 patients on CGH 749B and 4 patients on DFO complained of mild or moderate adverse events at the injection site. There was significantly prolonged release of desferrioxamine from CGH 749B ($T_{1/2}$ =36.3 h, n=9) and lower plasma concentrations (C_{max} = 0.95, 2.2, 2.4, 3.2 and 3.5µg/ml at doses of 2.5, 5.0, 7.5, 10.0 and 12.5 mg/kg respectively.) compared with the same patients receiving standard Desferal (terminal elimination $t_{1/2}$=7.59h), mean C_{max} = 9.2µg/ml. The proportion of the drug bound to iron (efficiency of chelation) prior to elimination in the urine was significantly higher with CGH 749B than following standard DFO. Furthermore, unlike standard DFO, metabolites of DFO were virtually undetectable, compatible with less drug being available for metabolism because of the higher chelating efficiency. The prolonged plasma $T_{1/2}$ with CGH749B was matched by a prolonged pharmacodynamic effect compared with standard DFO. Thus while cumulative urine iron excretion plateaud after 24-48h with standard DFO, continued urine iron excretion could be observed with CGH749B for 72-144h and total urine iron excretion with a CGH 749B dose of 6.7 mg/Kg was approximately equivalent to a standard dose of 40 mg/kg. Urine iron excretion with CGH 749B relative to standard DFO was linearly related to the dose as well as plasma area under the curve (AUC) ($p<0.006$). Based on these findings, modelling suggests that steady state plasma concentrations will be obtained following approximately 14 daily doses of CGH 749B. Phase II studies are in progress to determine whether this prediction is correct and to assess the tolerability of the depot formulation with repeat daily dosing.

New Approaches to the Non-Invasive Measurement of Iron Overload

Gary M. Brittenham, MD

Columbia University College of Physicians and Surgeons, New York, NY

A variety of new methods for the non-invasive measurement of iron overload are under development and evaluation. Clinical experience over the past decade has shown that the available indirect measures of iron overload (serum ferritin, transferrin saturation, urinary iron excretion after injection of deferoxamine) may often be misleading. The reference method for measurement of body iron stores is determination of the hepatic iron concentration in a biopsy sample (of adequate size, in the absence of cirrhosis or focal lesions of the liver) but the discomfort and risk of biopsy have prompted a search for non-invasive alternatives for the assessment of body iron. New approaches to the indirect evaluation of body iron are undergoing clinical appraisal. In the absence of erythroid hyperplasia, the concentration of circulating transferrin recep-tors seems to be decreased with iron overload. Measurement of the serum ferritin iron has been proposed as an improved means of estimating body iron. The underlying hypothesis is that serum ferritin protein produced in the acute phase response would have less iron than that produced in response to iron loading, thus avoiding the confounding effects of inflammation. A number of attempts to apply magnetic resonance imaging (MRI) to the measurement of iron in the liver and other tissues has been made using various instruments, magnetic field strengths, imaging protocols, and parameters (longitudinal [T1] and transverse [T2] relaxation times, signal intensity ratios of liver to muscle or other tissues in proton, T1-, T2- or T2*-weighted images). Several direct methods for measuring hepatic iron are being examined. The use of computed tomography (CT), with either a single or dual-energy technique, can potentially detect increased tissue iron by an increase in tissue X-ray absorption. The X-ray fluorescence of iron after exposure to a monochromatic X-ray beam or radioactive source has been considered for measurement in the skin, or, using a microprobe, in other tissues. Nuclear resonant scattering of X-ray is a method that uses gamma rays to raise ^{56}Fe to its first excited state and then counts the gamma rays produced by subsequent decay back to the ground state. At present, measurement of magnetic susceptibility to quantify paramagnetic ferritin and hemosiderin iron provides the only direct and noninvasive means of determining hepatic iron stores that has been calibrated and used extensively in studies of patients with iron overload. Only a limited number of the low-T_C (4.2°K) superconducting quantum interference device (SQUID) susceptometers used for these measurements are currently in use, in part because these instruments are expensive and technically demanding to operate. Efforts to develop alternative susceptometers with high-T_C (77°K) SQUID amplifiers and detectors or with room-temperature magnetoresistive sensors may make magnetic susceptibility measurement of hepatic iron more generally available.

Physiological and Pharmacological Properties of the Labile Iron Pool (LIP) as Revealed with a Fluorescent Metalo-Sensor in Living Mammalian Cells

Z. Ioav Cabantchik and William Breuer

Department of Biological Chemistry, Institute of Life Sciences, Hebrew University, Jerusalem, Israel

The labile iron pool (LIP) of mammalian cells is the transitory iron pool which contains the metabolic redox-active forms of the metal. It is defined operationally as the chelatable iron pool and viewed as chemically and topologically heterogeneous. LIP comprises both iron forms ((II and III) that are: 1. weakly bound to low and high molecular weight substances and 2. distributed among various cell compartments and 3. sensed and ultimately controlled by iron responsive proteins (IRPs) that regulate expression of transferrin (Tf)-receptors and of ferritin (Ft).

In order to obtain a direct measure of cell LIP levels in physiological conditions and pathological iron overload and during chelator treatment, we used fluorescent metalo-sensors targeted to defined cell compartments. The fluoresceinated-EDTA analog calcein (CAL) is a fluorescent probe which undergoes a swift fluorescence quenching upon stoichiometric binding of heavy metals such as iron and a fast de-quenching upon metal scavenging by high affinity binding iron chelators, ICH (1,2). CAL can be loaded into the cell cytosol via its permeant esterified CAL-AM precursor and into lysosomes by pinocytosis of dextran-calcein macromolecules. Within the cytosol, CAL binds iron from the LIP, forming the CAL-Fe complex, which is revealed as a rise in fluorescence signal evoked by application of rapidly cell-permeating ICHs. CAL-Fe is quantified with appropriate calibration curves and used for estimation of LIP (3). K562 cells grown in standard culture conditions contained 40-70 (M total iron, of which 0.4-0.8 (μM was associated with an ICH-accessible component defined as the major LIP (MLIP). An additional, but minor, LIP (mLIP) of ~0.2 (M cell iron, was revealed as an ICH-accessible component which is apparently in slow equilibrium with MLIP. MLIP levels were shown to be markedly affected: a. by the nature and extent of cell iron loads, such as in cells acutely or chronically exposed to inorganic iron salts (1-3), b. by oxidant and reductant stress (4,5), c. by the cytosolic iron buffering capacity, which could be modulated by ferritin in MEL cells engineered to express heavy chain-Ft subunits in an IRP-independent manner (6) and d. by treatment with ICHs (2,7). Based on these and other studies we concluded that continuous monitoring of cell CAL fluorescence can provide a dynamic and quantitative measure of a major component of the cytosolic cell LIP (3).

The CAL method of LIP detection allowed the quantitative analysis of ICH permeation into cells and its accessibility (rate and amplitude) to MLIP (2,7). These properties were shown to be dictated by the ICH's permeation coefficient and iron binding capacity (affinity and conc.) (7). Moreover, the principle of CAL quenching by iron and of CAL-Fe de-quenching by ICHs, were also adapted as extra-cellular devices for assessing: 1. trans-epithelial movement of ICHs; 2. secretion of iron from cells and 3. NTBI levels in sera of iron overloaded patients.

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Toxicological Evaluation of Iron Chelators

Philip Carthew, PhD

Medical Research Council, Toxicology Unit, University of Leicester, UK

The safety evaluation of iron chelators is a subject that needs particularly careful consideration, as the normal objectives of chronic toxicity may not be adequately addressed by a simple rodent study of the type normally carried out in the rat or mouse. This is because the question that is being asked is not just whether the compounds themselves exhibit toxicity, under conditions of a normal iron loading in vivo, but rather whether they will exhibit toxicity under conditions of iron overload.

Central to the safety evaluation of chelators is the selection of a species in which iron overload will produce the same major pathologies as are seen in man. With the recent identification of the Mongolian gerbil as the only rodent species which develops both hepatic fibrosis and cardiofibrosis in response to iron overload, it is particularly relevant to determine the response of this species to iron chelators, under conditions of iron overload. A preliminary study with the bidentate hydroxypyridinone iron chelator CP 94 has been carried out over a period of up to 20 weeks in the Mongolian gerbil, where the chelator and the iron have been administered over the same period, but not as an iron chelated complex. The initial inhibition of iron accumulation in the liver by CP 94, for the first 6 weeks was not maintained at 20 weeks. Furthermore, both at 6 and 20 weeks there was a significant increase in the levels of cardiac iron in animals treated with iron and chelator. This was accompanied by an increase in cardiofibrosis at 20 weeks and also in liver damage to produce micronodular cirrhosis. Although the increased cardiotoxicity was related to increased levels of cardiac iron, the increase in hepatotoxicity occurred in the absence of any increase in liver iron levels above the control levels found with iron alone treatment. The implications of this study are that the gerbil is suitable as an experimental species, able to detect tissue redistribution of iron under chelating conditions, and produces the same pathology end points seen in man during iron overload.

Metal Chelation, Ischemia/Reperfusion and Diabetes

John W. Eaton, PhD

Department of Pediatrics, Baylor College of Medicine, Houston, TX

Delocalization of transition metals, especially iron and copper, can occur in a number of clinical disorders. One under consideration here—ischemia/reperfusion [I/R]—is also associated with enhanced production of activated oxygen species, leading to the possibility of metal-catalyzed free radical reactions. The importance of the latter is indicated by numerous observations that administration of chelators such as desferrioxamine (which has high affinity for iron but also will bind other metals such as copper) will moderate organ damage arising from experimental I/R. Despite years of research in this area, the source(s) of delocalized transition metal (iron and, possibly, copper) and of activated oxygen production remain largely unknown. Nonetheless, it does appear that transition metal chelating drugs may have some future application in therapy of I/R injury. This conclusion should be tempered by the fact that, in many experimental models, chelators must be present at the time of reperfusion and such precise timing may be impossible in many clinical circumstances.

The possible involvement of chelatable transition metals in diabetic vasculopathy and peripheral neuropathy is indicated by recent observations in experimental rodent models of diabetes that several transition metal chelating drugs will, *in vivo*, (i) restore normal neuronal blood flow, (ii) reverse defective nerve conduction velocity and, (iii) *in vitro*, normalize defective acetylcholine-mediated arterial relaxation (which is dependent on nitric oxide [NO] produced by the endothe-lium). As a possible explanation for these salubrious effects of chelators, we hypothesized that, upon reaction with sugars such as glucose and fructose, glycated derivatives might form which were capable of binding transition metals such as copper and iron and, perhaps, support reduction/oxidation reactions involving the bound metal. Inasmuch as the long-lived proteins of the internal elastic lamina (collagen and elastin) are heavily glycosylated in diabetics, should these form 'glycochelates' *in vivo* it might lead to the genesis of a metal-rich barrier immediately below the endothelium which would catalytically decompose NO compounds. In partial support of this hypothesis: (1) Upon glycation (with either glucose or fructose), the three proteins we have investigated—albumin, gelatin (a soluble fragment of collagen) and elastin—all acquire a substantial binding affinity for both iron and copper. (2) When glycated proteins with bound metal are incubated with ascorbic acid, substantial metal-catalyzed oxidation of ascorbate ensues. (3) Perhaps more importantly, these glycochelates—especially those containing copper—effect the rapid catalytic decomposition of nitrosothiols (such as nitrosocysteine), the likely transport form of NO. In vivo, such metal-catalyzed decomposition of nitrosothiols could impair endothelium-dependent vascular relaxation, producing a state of diminished peripheral blood flow. The possible *in vivo* accumulation of such 'glycochelates' within the internal elastic laminae of arteries may help explain the diminished peripheral neural blood flow and decreased nerve conduction velocity in diabetics. Should this hypothesis prove to be true, administration of transition metal chelators may hold substantial promise for the alleviation of diabetic vasculopathy and peripheral neuropathy.

A) Exacerbation of Fungal Infections by Siderophores.
B) The Role of Iron in HIV Infection.

Johan R. Boelaert, MD

Algemeen Ziekenhuis St-Jan, Brugge, Belgium

A) Deferoxamine (DFO) therapy does not influence the overall incidence of bacterial infection but it enhances the risk of infection by *Yersinia enterocolitica* or *Y. pseudotuberculosis*. More importantly, DFO increases the risk of zygomycosis ("mucormycosis"). Our registry (1,2) disclosed 49 dialysis patients on DFO who developed this infection (generalized in 49%, fatal in 90%). DFO and also its ferrated form FO aggravated experimental infection caused by the zygomycete fungus *Rhizopus* (3). Addition of 0.01 μM^{55} Fe-FO to a serum-containing medium resulted in radioiron accumulation by and growth stimulation of *Rhizopus* and *Cunninghamella* (2,4), indicating that this infection is siderophore-mediated. The enhanced risk of DFO-associated zygomycosis in dialysis patients as opposed to non-uremic hematological patients is due to the prolonged FO serum half-life in uremia (5). The hydroxypyridinone chelators CP20 & CP94 do not enhance the risk of zygomycosis (6). Although FO may also somewhat enhance the in vitro growth of the non-zygomycete fungi *A. fumigatus* or *C. neoformans*, this does not result in clinical infection (2).

B) During HIV infection, particularly in its more advanced stage, iron accumulates in macrophages, microglia, endothelial cells and myocytes, resulting in an iron burden in bone marrow, brain, muscle, liver and spleen. **Main causes** of this iron excess are the chronic inflammatory response that involves iron withholding, and blood transfusion (7). **Consequences** of this iron excess are: 1° increased oxidative stress, activation of NFkB and enhanced transcription of HIV (8). Accordingly, patients with the haptoglobin 2-2 phenotype, who accumulate more iron, have higher HIV-1 RNA plasma levels and a significantly worse survival than haptoglobin 1-1 or 2-1 patients (9); 2° impairment in function of granulocytes and macrophages and in cell-mediated immunity; 3° stimulated growth of microorganisms, including AIDS-opportunists; 4° an increased risk of HIV-related tumours, such as Kaposi's sarcoma. Spindle cells from these tumours in culture grow better in the presence of iron and are growth-inhibited in the presence of iron-chelators (10). As a **practical consequence**, studies are needed on the possible usefulness of an adjunctive anti-HIV strategy that prevents iron loading by: 1° targetting patient populations at special risk of iron loading, e.g. haptoglobin 2-2 patients (9); 2° restricting iron intake through parenteral, alimentary and respiratory routes (7); 3° using drugs such as chloroquine that may limit deposition of iron in the reticulo-endothelial system (11); 4° prudently using an iron chelator. One study in thalassemics showed that HIV infection showed less progression when the patients were treated with an effective dose of DFO (12).

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Iron Chelation as a Therapeutic Strategy for the Treatment of Malaria

Victor R. Gordeuk, MD

The George Washington University Medical Center, Washington, DC

Malaria is one of the major health problems globally and an urgent need for the development of new antimalarial agents faces the scientific community. A considerable number of iron (III) chelators, designed for purposes other than treating malaria, have anti-malarial activity *in vitro*. Examples include the hexadentate trihydroxamic acid, desferrioxamine, and the bidentate hydroxypyridinone, deferiprone. Such chelators enter the erythrocytic parasite and bind labile iron within the parasitic cytosol. Their mechanism of action appears to involve the withholding iron from vital metabolic pathways of the intraerythrocytic parasite, and it is also possible that they might enhance host macrophage function. Certain iron (II) chelators also have anti-malarial activity, but the mechanism of action appears to be the formation of toxic complexes with iron rather than the withholding of iron. Several of the iron (III) chelating compounds have anti-malarial activity in animal models of plasmodial infection. Iron chelation therapy with parenteral desferrioxamine has clinical activity in both uncomplicated and severe malaria in humans. Oral deferiprone does not have activity in animals or humans, possibly due to limited peak plasma levels and rapid clearance from the plasma. Efforts to design iron chelators specifically for the treatment of human malaria would seem to be in order.

Iron Metabolism in Health and Disease: Problems and Perspectives

Philip Aisen, MD

Albert Einstein College of Medicine, Bronx, NY

Prior to two decades ago studies of iron metabolism and its chemistry were largely descriptive. The modern era may be said to have begun with determination of the crystal structure of horse spleen ferritin, elucidation of the major features of the transferrin-to-cell cycle in iron metabolism, and recognition of the regulatory events in expression of the transferrin receptor and ferritin, proteins controlling the uptake and storage of iron by cells. Accelerating progress in more recent years has led to identification of the long-sought hemochromatosis (HFE) gene, the intestinal iron-transporter gene (Nramp-2), and the functional association of the hemochromatosis gene product with ß2-microglobulin and the transferrin receptor. Still awaited are the 3-D structures of the transferrin receptor and the transferrin-transferrin receptor complex.

Inevitably, answers to long-standing questions have generated new questions, unforeseeable a few years ago and not yet answered. What is the normal function of the hemochromatosis gene, and what are the roles of ß2-microglobulin and the transferrin receptor in this function? What are the roles of cytokines and the immune system in regulation and function of proteins of iron metabolism? How and in what form is iron exported from cells where it is stored, recovered from senescent or damaged red cells, or absorbed, and how is such export regulated? Questions abound, and inevitably provoke research and new understanding.

Progress in the clinical realm has been less dramatic. Reliable and economically feasible noninvasive assessment of iron overload is still problematic. It is not clear whether iron overload and iron toxicosis parallel each other in more or less linear fashion before, during and after treatment. Evaluating efficacy of chelation therapy is not entirely simple and straightforward. The long-sought oral chelator that is safe and effective is not yet at hand, although leads toward this goal continue to appear in encouraging manner. Disorders of iron metabolism are among the most common of human ills, and their effective management remains a persistent concern of modern molecular medicine.

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